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(54) Title: BLOCKERS OF ION CHANNELS AND METHODS OF USE THEREOF

(57) Abstract

This invention relates to methods of treatment of diseases and various other undesirable bodily states which involve the excessive, inappropriate, and/or prolonged activity of voltage-activated ion channels, and are ameliorated by administration of compounds which block said channels. The methods involve the administration to mammals of compounds which block one or more classes of voltage-activated sodium and/or calcium ion channels, in a manner operationally defined herein as "use-dependent". Specific examples of conditions treatable by compounds of the invention include but are not limited to ischemic brain disease, ischemic heart disease, epilepsy, and amyotrophic lateral sclerosis (LAS).

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TITLE: BLOCKERS OF ION CHANNELS and METHODS OF USE THEREOF

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BACKGROUND OF THE INVENTION

1. FIELD OF THE INVENTION

The present invention pertains to ion channel blockers and methods of treatment and pharmaceutical compositions.

2. BACKGROUND

A. Properties Of Voltage-Activated Ion Channels

Voltage-activated sodium ("Na") and calcium ("Ca") channels of electrically excitable cells are currently understood as being protein molecules which are embedded in the lipid bilayer membranes of cells [Hille, B (1992) Ionic Channels of Excitable Membranes, 2nd Edition, Sunderland, MA., Sinauer Assoc., pp. 59-114; Goldin, S.M. (1986) *Molecular Level Characterization of Ion channels*, in The Heart & Cardiovascular System: Scientific Foundations New York, Raven Press, pp. 537-560]. Said ion channels play a central role in regulation of a wide range of cellular functions in mammals. The opening and closing of a pathway comprising a "pore" within the ion channel molecule regulates the movement of Na and Ca across the cell membrane; this in turn alters the electrical potential across the cell membrane. In certain instances the movement of said ions through the channels may alter the ion concentrations within or outside the cell.

As a result of the ability of said ion channels to produce voltage-activated transmembrane movement of Ca and Na, they are responsible for the electrical signals which underlie the rapid flow of information within the brain, and from the brain to the spinal cord [Catterall, W.A. (1992) Physiol. Rev. **72**, S15-S48]; Bean, B.P. (1989) *Ann. Rev. Physiol.* **51**: 367-384; Hess, P. (1990) Ann. Rev. Neurosci. **13**:337-56]. They produce and propagate the electrical signals in nerve cells which initiate and regulate muscle contraction [Catterall (1992) *ibid.*; Bean (1989), *ibid.*]. Within the heart, voltage-activated Na and Ca channels in the AV and SA nodes, the Purkinje cell

conduction network, and the atrial bundle branches are responsible for insuring that cardiac muscle contracts in a coordinated and properly timed manner to effectively pump blood through the circulatory system [Katz, A.M. (1977) Physiology of the Heart, pp. 107-119, 209-256; Catterall (1992) *ibid.*; Bean (1989), *ibid.*].

In addition to the aforementioned roles of said channels, which encompass electrical signals governing cellular communication on the timescale of milliseconds to seconds, voltage-activated Ca channels regulate processes which occur on a longer time frame of minutes to hours. These processes include but are not limited to secretion of peptide hormones such as growth hormone from the pituitary gland [Tse et al. (1993) Science 260, 82-84; Chang, J.P. et al. (1988) Endocrinology **123**, 87-94; Tan, K.N., and Tashjian, A.H. (1984) J. Biol. Chem. **259**: 418-426] and insulin from pancreatic beta cells [Larner, J. (1985) in *The Pharmacological Basis of Therapeutics, 7th Ed.*, Goodman, L.S. and Gilman, A.G. eds., New York, MacMillan, pp. 1490-1503]; catecholamines from the adrenal medulla [Landsberg, L. and Young, J.B. (1987) in Harrington's Principles of Internal Medicine, 11th Ed., Braunwald et al. eds., New York, McGraw-Hill, pp. 358-370; Neher, E. and Zucker, R.S. (1993) Neuron **10**, 21-30]; and digestive enzymes from the pancreas [Muallem, S. (1989) Ann. Rev. Physiol. **51**: 83-105].

Excessive and/or inappropriate conduction of ions through said channels produce harmful consequences in a wide variety of disease states, exemplified but not limited to epilepsy [Porter, R. J. (1989) Epilepsia 30, Suppl. 1, S29-S34; Rogawski, M.A., and Porter, R.J. (1990) Pharmacol. Rev. 42, 224-270], stroke [Choi, D.W. (1990) Cerebrovasc. & Brain Metab. Revs. 2; 105-147]; cardiac arrhythmias [Zipes, D.P. (1984) in Braunwald, Heart Disease: A Textbook of Cardiovascular Medicine, Phila., SaundersCo., pp. 605-647; Bigger, J.T., and Hoffman, B.F. (1985) in *The Pharmacological Basis of Therapeutics, 7th Ed.*, Goodman, L.S. and Gilman, A.G. eds., New York, MacMillan, pp. 748-783]; chronic pain [Gracely, R.H. et al (1992) Pain **51**,

175-194; and hyperkalemic periodic paralysis [Cannon et al. (1991) Neuron 6, 619-626]. In certain other diseases, the underlying causes of the disorders are not as yet well defined, but relief is known to be provided by administration of compounds which act by attenuating the activity of said channels. A well-known example of this is essential hypertension, which can be alleviated by administration of blockers of the voltage activated Ca channels which govern the contractility of vascular smooth muscle [Triggle, D. J. (1990), in, "Cardiovascular Pharmacology", ed. M. Antonaccio, 3rd ed., Raven Press, N.Y., pp. 107-160].

Voltage-activated Na and Ca channels cycle through a series of conformational states in response to changes in membrane potential which occur during both normal and pathophysiological channel activity [Triggle et al. (1989) Medicinal Res. Rev. **9**, 123-180; Siegelbaum, S.A., and Koester, J. (1991) in Principles of Neural Science, 3rd Ed., Kandel, E.R. et al., eds., Norwalk CN, Appleton & Lange, pp. 66-79]. These conformational states, which correlate with particular functional states of said channels, are most simply schematized below:

In response to depolarization of the cell membrane to, typically, -10 to +20 mV, an ion channel is more likely to be found in the open state or in the closed, inactivated state than in the closed, activatable state most favored for a metabolically "healthy" cell which is most frequently found at the hyperpolarized, "resting" membrane potential of, typically -60 to -100 mV.

Certain pathophysiological circumstances produce sustained and/or repetitive depolarization of the cell membrane, as occurs in acute disorders including but not limited to brain ischemia resulting from stroke, cardiac arrest [Choi, D.W. (1990) *Cerebrovascular and Brain Metab. Rev.* **2**, 105-147; Meldrum, B.S.(1990) Cerebrovasc. Brain Metab. Rev. 2, 27-57] and in chronic disorders, among them epilepsy [Porter, R. J. (1989) Epilepsia 30, Suppl. 1, S29-S34; Rogawski, M.A., and Porter, R.J. (1990) Pharmacol. Rev. 42, 224-270]; head trauma [Marshall, L.F. (1990) Curr. Opin. in Neurol. and Neurosurg. **3**, 4-9]; amyotrophic lateral sclerosis (LAS) [Appel, S.H. (1993) Trends Neurosci. **16**, 3-5] , and Huntington's disease [Choi, D.W. (1988) Neuron 1, 623-634].

Said excessive and/or inappropriately timed depolarization of the cell membrane produces excessive cellular Ca entry; the elevation of free cellular Ca levels in turn leads to cellular injury and destruction [Choi,D.W. (1988) Neuron 1, 623-634; Choi, D.W. (1990) Cerebrovasc. & Brain Metab. Revs. **2**; 105-147; Marshall, L.F. (1990), *ibid.*]. In other disorders, including but not limited to cardiac arrhythmias [Zipes, D.P. (1984) in Braunwald, Heart Disease: A Textbook of Cardiovascular Medicine, Phila., SaundersCo., pp. 605-647; Bigger, J.T., and Hoffman, B.F. (1985) in *The Pharmacological Basis of Therapeutics, 7th Ed.*, Goodman, L.S. and Gilman, A.G. eds., New York, MacMillan, pp. 748-783], **chronic pain (ref)**, Parkinson's disease Parkinson's Disease [Miller, W.C., and DeLong, M. (1987) in Carpenter, M.B. and Jayaraman, A, eds. The Basal Ganglia, vol.II, New York, Plenum Press, pp. 415-427; Mitchell, I.J. et al., (1989) Neurosci. **32**, 213-226] , and hyperkalemic periodic paralysis

[Cannon et al. (1991) Neuron 6, 619-626], excessive depolarization-induced activity of said voltage-activated ion channels produces adverse physiological consequences in the excitable cells of, respectively, cardiac, nerve, or muscle tissue. In the case of cardiac arrhythmias, the result is inappropriately timed contractions of cardiac muscle with potentially lethal consequences. In the case of chronic pain, it is uncontrolled activation of the neuronal pain signalling pathways. In the instance of Parkinson's disease, excessive nerve activity leads to pathological changes in the cellular activity in the basal ganglia of the brain of dopamine-deficient patients, producing movement disorders. Finally, in the case of hyperkalemic periodic paralysis prolonged activation of abnormally functioning voltage-gated Na channels of muscle cells produces paralysis.

B. **Blockers Of Voltage-Activated Na And Ca Channels Controlling Glutamate Release**

As described herein, the molecular targets of a large subset of compounds of the invention are the voltage-activated ion channels which govern the release of glutamate and other neurotransmitters. In particular, **two classes of voltage-sensitive ion channels play a central role in governing presynaptic glutamate release**:

(a) **Voltage-activated sodium ion-channels** in axons and nerve terminals initiate glutamate release by propagating a depolarizing stimulus to the sites of vesicular release [Zivin, J.A. and Choi, D.W. (July, 1991) Sci. Amer. **265**, 36-43].

(b) **Presynaptic voltage-activated calcium ion-channels** at the release sites are opened in response to this depolarization. The elevation of cytoplasmic Ca levels in the nerve terminal initiates the process of exocytosis of glutamate [Meldrum, B. (1990) Cerebrovascular and Brain Metab. Rev. **2**, 27-57; Choi, D.W. (1990) Cerebrovascular and Brain Metab. Rev. **2**, 105-147].

The neuron specific Type II subclass of voltage-activated Na channels, found in nerve axons and nerve terminals [Westenbrook, R.E. et al., Neuron **3**, 695-704; Catterall, W.A. (1992) Physiological Rev. **72**, S15-S41], is claimed to be the most directly involved of all the Na channels in initiating release of glutamate and other neurotransmitters [Leach, M.J. et a. (1986) *Epilepsia* 27:490-497]. Antagonists of the neuron-specific type II subclass of voltage-gated Na channels are neuroprotective [Stys, P. K., S. G. Waxman, and B. R. Ransom (1992) *J. Neurosci.* **12**, 430-439; Graham, S.H., J. Chen, F.H. Sharp, and R.P. Simon (1993) J. Cereb. Blood Flow and Metab. **13**: 88-97; Meldrum, B.S., et al. (1992) *Brain Res.* **593**, 1-6; Wahl, F. et al (1993) *Eur. J. Pharmacol.* **230**: 209-214] . In particular, several antagonists of said Na channels are among those that have been shown to block glutamate release [Leach, M.J. et a. (1986) *Epilepsia* **27**:490-497; Benoit, E., and Esconde, D. (1991) *Pflugers Arch.*

1991, 419-22; Graham, S.H., J. Chen, F.H. Sharp, and R.P. Simon (1993) *J. Cereb. Blood Flow and Metab.* **13**: 88-97]. The following discussion serves to explain, at least in part, the connection between block of type II Na channels and inhibition of glutamate release.

As shown in **Figure 1**, in focal ischemic situations such as stroke, sustained hypoxia in the "core region" results from occlusion of the blood supply by a clot [Zivin, J.A. in Protection of the Brain from Ischemia, Weinstein, P.R. and Faden, A.I., eds., Baltimore, Williams and Wilkens, 1990]. As hypoxia develops, ATP depletion [Shimizu, H. et al.(1993) Brain Res. **605**, 33-42] leads to an inability of the active Na and K-ion pump, the Na,K-ATPase, to maintain the ion gradients which generate the normal membrane potential of resting nerve cells. As the cell depolarizes and reaches the threshold for action potential firing, Na channels are activated. Hyperactivity of Na channels has at least 2 important pathophysiological consequences leading to excessive glutamate release:

(a) trains of action potentials may invade the penumbra from the core [Figure 7b] extending the excessive glutamate release, and hence the neuronal damage, to regions well beyond the ischemic focus;

(b) ion gradients in axons and nerve terminals are further depleted, generating an increased energy demand to re-establish Na and K gradients and, secondarily, for reuptake and resynthesis of neurotransmitters released as a result of hyperactivity; and, the resulting rise in intracellular [Na] and concomitant membrane depolarization causes damaging levels of Ca to enter the neuron through reverse operation of the electrogenic Na/Ca exchanger. These secondary events can lead to further excessive release of glutamate.

Stys *et al* [Stys, P. K., S. G. Waxman, and B. R. Ransom (1992) J. Neurosci. **12**, 430-439] recently reported the development of Na channel hyperactivity in anoxia of central white matter and demonstrated *in vitro* the neuroprotective effect of the Na channel blockers tetrodotoxin (TTX) and saxitoxin (STX). Their experiments substantiate the pathophysiological role of mechanisms (a) and (b) described above.

A concrete demonstration of the neuroprotective efficacy of glutamate release inhibitors which act by blocking Type II Na channels comes from recently published *in vivo* and *in vitro* studies of a glutamate release blocker, BW1003C87 (Burroughs-Wellcome) [Graham, S.H., J. Chen, F.H. Sharp, and R.P. Simon (1993) J. Cereb. Blood Flow and Metab. **13**: 88-97; Meldrum, B.S., et al. (1992) Brain Res. **593**, 1-6]. This compound is reported to possess an acceptable therapeutic index from the standpoint of CNS toxicity and cardiovascular safety. However, BW1003C87 reportedly possesses potent antifolate activity, unrelated to its ability to block Na channels, which causes anemia at neuroprotective dose levels and may limit its clinical utility [Graham, S.H., J. Chen, F.H. Sharp, and R.P. Simon (1993) J. Cereb. Blood Flow and Metab. **13**, 88-97]. This is one of several examples demonstrating that, although the therapeutic efficacy of glutamate release blockers in animal models has been clearly established, there is clearly a need for novel neuroprotective glutamate release blockers with an acceptable safety profile.

Regarding the involvement of presynaptic voltage-activated Ca channels in glutamate release, it is first necessary to summarize current state of knowledge of Ca channel diversity and pharmacology. It is generally accepted that there are at least 4 subclasses of Ca channels (T,N, P and L) that differ in their pharmacology, location in neuronal and non-neuronal tissues, and physiological properties [Bean, B.P. (1989) Ann. Rev. Physiol. **51**, 367-384., Mintz, I. M., M. E. Adams and B. P. Bean (1992) Neuron **9**, 85-95]. Pharmacological criteria have been used to distinguish among these channel subclasses: L-type Ca channels are particularly sensitive to dihydropyridine Ca

antagonists such as nifedipine and nimodipine, and N-type Ca channels are specifically blocked by the cone snail peptide, ω-conotoxin-GVIA [Bean, B.P. (1989) Ann. Rev. Physiol. **51**, 367-384]. Recently, a new subclass of presynaptic Ca channels controlling neurotransmitter release, termed "Q-type", has been identified [Zhang, J.-F. at al. (1993) Neuropharmacol. 32, 1075-1088]. Q-type channels are closely related to P-type Ca channels: P- and Q-type Ca channels are insensitive to dihydropyridine Ca antagonists or w-conotoxin-GVIA. However, both P- and Q-type channels are specifically blocked by the spider venom peptide ω-aga-IVA, the former subclass being more sensitive to this toxin than the latter.

Certain venom peptides which block N-type Ca channels and result in block of catecholamine release, among them the cone snail peptide ω-conotoxin M-VII-A ("SNX-111'), have shown efficacy in animal models of brain ischemia [Valentino, K. et al. (1993) Proc. Nat. Acad. Sci. USA 90, 7894-7]. SNX 111 is currently in human clinical trials for prevention of ischemic brain damage (ref). SNX 111, which is hypothesized to interact with the ion-selectivity filter [Boland, L., Morrill, J., and Bean, B. (1994) J. Neurosci., in press] but in a reverse use-dependent manner enhanced by repetitive depolarization, as revealed by brain slice studies [Wurster, S., and Dooley, D.J. (1993) Abs., Soc. for Neurosci. **19**, 1750], demonstrates side effects, including profound hypotension, which may seriously limit its use in treatment of focal ischemia [Xue, D. et al. (1993) Abs., Soc. for Neurosci. **19**, 1643]. We demonstrate herein that the chemical, pharmacological, and mechanistic properties of compounds of the current invention, which act at the ion selectivity filter but, in contrast to SNX 111, exhibit positive use-dependence, are advantageous over those of said peptide for prevention of focal ischemic neuronal damage.

The particular attractiveness of blockers of glutamate release for therapy in situations where brain damage is attributable to both focal and global ischemic neuronal damage is illustrated in **Figure 2** and the following discussion. NMDA antagonists are

highly effective in animal models of focal cerebral ischemia [Albers, G.W., M.P. Goldberg, and D.W. Choi (1989) Annals of Neurol. **25**, 398-403], but are reportedly much less effective in models of global cerebral ischemia [Buchan, A., Li, H., and Pulsinelli, W.A. (1991) *J. Neurosci.* **11**, 1049-1056; Choi, D.W. (1990) *Cerebrovascular and Brain Metab. Rev.* **2**, 105-147]. In contrast, non-NMDA antagonists such as NBQX are highly effective in rat models of global cerebral ischemia [Buchan, A., H. Li, S. Cho and W. A. Pulsinelli (1991) Neurosci. Lett. **132**, 255-258], but are apparently much less effective in models of focal cerebral ischemia [Meldrum, B.S.(1990) Cerebrovasc. Brain Metab. Rev. **2**, 27-57].

Figure 2 illustrates the generally accepted key steps in the cascade of events that lead to neuronal cell death in ischemia. NMDA antagonists prevent nerve cell death resulting from the events depicted in the lefthand limb of the diagram. Antagonists of non-NMDA receptors block cell death resulting from the events shown on the righthand limb of the diagram. A blocker of glutamate release, by acting at an earlier stage of the process, prevents excessive activation of both NMDA and non-NMDA receptor subclasses, and thus combines the advantages of both NMDA and non-NMDA receptor antagonists. This has recently been demonstrated in *in vivo* studies of the glutamate release blocker BW1003C87 [Graham, S.H., J. Chen, F.H. Sharp, and R.P. Simon (1993) J. Cereb. Blood Flow and Metab. **13**: 88-97; Meldrum, B.S., et al. (1992) Brain Res. **593**, 1-6]. Said *in vivo* studies clearly demonstrated that BW1003C87 is as effective in models of global ischemia as non-NMDA antagonists, and is as effective in models of focal ischemia as noncompetitive NMDA antagonists such as MK 801 and CNS 1102.

A compound that is efficaceous in both focal and global ischemia should be ideally suited to acute treatment of brain damage resulting from high risk cardiovascular surgery. For example, in coronary artery bypass surgery, subsequent neurological deficits have been attributed not only to interruption of the brain's blood supply from

the heart, which constitutes a global ischemic insult, but also to small clots (microemboli) that produce multiple focal cerebral lesions [Shaw, P.J. et al. (1987) Stroke **18**, 700-707]. It is an object of the current invention to create compounds which, as a result of their ability to block release of glutamate, are efficaceous in global and focal cerebral ischemia and which have an acceptable safety profile.

Excessive and/or inappropriate dopamine and norepinephrine release, which also occurs in hypoxia/ischemia, may mediate brain injury as well [Globus, M.Y.-T., M.D. Ginsburg et al. (1988) Neurosci. Lett. **91**, 36-40; Globus, M.Y.-T., M.D. Ginsburg et al. (1989) J. Cereb. Blood Flow & Metab. **9**, 892-896.]. Thus, early intervention with compounds that block release of catecholamines and possibly release of other transmitters, in addition to release of glutamate, should be a more effective means of preventing hypoxic/ischemic brain injury than blockade of glutamate release alone.

Although the therapeutic benefits of compounds which act in a use-dependent manner are predicted by the prior art, and compounds exist which are therapeutically effective use-dependent blockers of certain subclasses of said Ca and/or sodium channels, their discovery to date has relied largely on empirical methods of drug screening and medicinal chemistry commonly practiced by the pharmaceutical industry. A need clearly exists for additional methods of identifying compounds that act as use-dependent blockers of said channels. Naturally occurring compounds which block Na channels, such as tetrodotoxin and saxitoxin [Hille, B (1992) Ionic Channels of Excitable Membranes, 2nd Edition, Sunderland, MA., Sinauer Assoc., pp. 59-67] do not block said ion channels in a use-dependent manner as operationally defined herein. The insufficiency of use-dependence in blockade of ion channels limits their therapeutic utility, because said toxins indiscriminately block said ion channels whether or not they are in the open, activatable, or closed conformations. This indiscriminate blockade of said channels, whether or not they are in cells functioning in a normal manner not

requiring channel blockade, results in untoward toxicity, which in the cases of tetrodotoxin and saxitoxin includes respiratory paralysis [Narahashi, T. (1975) in The Nervous System, Vol. 2, Tower, D.B.,ed., New York, Raven Press, pp. 101-110]; Wiberg, G.S. (1960) Appl. Pharmacol. 2, 607;. This is also the case for naturally occurring compounds, among them cone snail ω-conotoxin peptides and spider venom peptides including the ω-agatoxins (see "Detailed description of invention").

SUMMARY OF THE INVENTION

The present application describes and claims novel, therapeutically useful compounds and methods for identifying such compounds which exhibit the property of "use-dependent" block of ion channels which conduct cations across the cell membrane. These compounds are particularly well suited to treatment of disorders involving repetitive, persistent or inappropriate depolarization of the cellular membrane potential.

Specifically, the present invention relates to methods of treating mammalian diseases which are associated with increased frequency or duration of depolarization of cells, comprising the administration of a therapeutically effective amount of a compound which inhibits said cells' voltage-activated ion channels preferentially when said channels are activated by repetitive or sustained depolarization by interacting with the SS1/SS2 selectivity filter of said ion channels. The present invention further relates to compounds and methods of identifying compounds which 1) preferentially inhibit cellular ion channels activated by repetitive or sustained depolarization and 2) interact with the SS1/SS2 selectivity filter of said ion channels.

These compounds can preferentially inhibit the excessive influx of cations through voltage-activated sodium and calcium channels of cells that experience increased frequency or duration of depolarization while exerting minimal affects on said sodium and calcium ion-channel activity in normal cells, which are relatively more likely to be found in the hyperpolarized state. Specifically, they block the excessive and/or inappropriate entry of cations into cells affected by pathophysiological circumstances which produce sustained and/or repetitive depolarization of the cell membrane while not affecting significantly the function of normal cells. As such, these compounds are particularly advantageous in treating diseases and/or disorders caused by cells which experience increased frequency or duration of depolarization. These

diseases and disorders include but are not limited to brain ischemia resulting from stroke, cardiac arrest and head trauma, epilepsy, amyotrophic lateral sclerosis (LAS) and Huntington's disease.

In particular, a preferred embodiment of the present invention offers a significant advantage over compounds currently available for the treatment of the diseases and/or disorders mentioned above. This advantage relates to the ability of a subset of the invention's compounds to block both voltage-activated calcium and sodium channels due to the specific interaction of the compounds with a site contained within both classes of channels. This site, known as the SS1/SS2 ion-selectivity filter, shares a high degree of structural and functional homology in calcium and sodium channels, allowing interaction and blockage of both channel types by the same compounds. Under certain pathophysiological circumstances which produce sustained or repetitive depolarization of the cell membrane, it is highly desirable to administer a therapeutic agent which can block the entry of cations through both classes of channel as opposed to only one class of channel. This eliminates the need to administer two separate and different drugs to block the two types of ion channels.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates key features of the pathophysiology of focal ischemic damage in stroke.

Figure 2 illustrates a cascade of biological events initiated by brain ischemia and leading to nerve cell death.

Figure 3 is a diagram of the arrangement in the cell membrane of the α subunits of a voltage-activated Ca channel and Na channel, illustrating the structural homology between said channels.

Figure 4a and 4b illustrate a structural model of voltage-activated Na and Ca channels.

Figure 4c displays mutations in the SS2 regions of a voltage-activated Ca channel which affect the ion-conducting behavior of said channels.

Figure 5 illustrates the ability of compounds of the invention to competitively inhibit the binding of radiolabeled [^{3}H]-saxitoxin to the SS1/SS2 ion-selectivity filter region of voltage activated Na channels of rat brain nerve terminals (synaptosomes). Refer to **Example 5**.

Figure 6a provides protein sequence data of the SS1/SS2 regions of the internal repeat I of a variety of voltage-activated Ca channel and Na channel clones.

Figure 6b depicts the amino acid sequence of the SS2 region of voltage-activated Na channels, illustrating the differences in the sequences between tetrodotoxin-sensitive and tetrodotoxin-insensitive forms of the channel.

Figure 7 illustrates structural similarities between tetrodotoxin and two compounds of the invention, CNS 1145 and CNS 1237.

Figures 8a and 8b illustrate the use dependent actions of compounds of the invention on nerve terminal Ca channels which results in the ability of said compounds to accelerate the decay of glutamate release from brain nerve terminals as measured by rapid superfusion. It further contrasts this property with the behavior of cone snail and spider venom blockers of said channels, demonstrating that glutamate release blockade is relieved by sustained depolarization. Refer to **Example 4**.

Figure 9 illustrates the ability of a compound of the invention to block voltage-activated Na channels expressed in CHO cells, in a use-dependent manner enhanced by repetitive depolarization of the cell membrane. It contrasts this property with the lack of use dependence block of said channels by of a compound of the prior art, tetrodotoxin. Refer to **Example 2.**

Figure 10a and 10b illustrates another manifestation of use-dependent block of said voltage-activated Na channels by two compounds of the invention: the ability of depolarization to enhance the rate of block of Na channels by said compounds. Refer to **Example 3.**

Figure 10c illustrates the lack of depolarization-dependent enhancement of the rate of block of said voltage-activated Na channels by a compound of the prior art, tetrodotoxin. Refer to **Example 3.**

Figure 11 demonstrates block by the cone snail venom peptide ω–conotoxin MVIIC of glutamate release from brain nerve terminals as measured by rapid superfusion.

Figure 12 illustrates the ability of a compound of the invention, CNS 1237, to provide neuroprotection in an animal model of stroke, the rat middle cerebral artery (MCAO) occlusion model. Refer to **Example 9**.

Figure 13 displays the ability of compounds of the invention to reduce the size of kainate-induced lesions of rat brain (refer to **Example 10**).

Figure 14 provides the amino acid sequences of the SS1/SS2 ion selectivity filter domain for a representative group of voltage-activated Na and Ca channels.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS OF KEY TERMS:

Note: in some instances, the operational definitions of the terms used herein may be somewhat more specialized than the broadest uses of the terms as may on occasion be employed in the scientific literature.

administration: delivery of a substance to the individual in need of treatment. For example, a substance (in particular a compound of the invention) may be administered intravenously or orally.

compound: a homogenous substance of defined or definable chemical structure. Examples of compounds of relevance are the substituted guanidine compounds of the invention such as CNS 1237, substituted guanidine toxins outside the scope of the current invention such as saxitoxin and tetrodotoxin, and peptides outside the scope of the current invention such as the ω-conotoxins GVIA and MVIIA.

depolarization: reduction of the membrane potential of a cell from values found in a cell in the "resting, hyperpolarized" state. Said values are normally about -70 mV to -90 mV (negative on the inside surface of the cell membrane). When a cell fires action potentials due to the opening of voltage-activated Na or Ca channels, the depolarization is usually robust, reaching values of, typically, about -10 to +20 mV. In ischemic and/or hypoxic situations, the depolarization is typically more gradual and smaller in magnitude, declining to values of, not atypically, about -60 to -30 mV. When said values are reached, a pathophysiological condition of abnormal excitability and concomitant dysfunction of the cell may occur, adversely affecting the surrounding tissue and the health of the individual.

depolarization, increased frequency of: a state of an excitable cell in which more frequent depolarization occurs. The use of this term that is of greatest relevance to the current invention involves the more frequent reduction of the membrane potential. Each depolarizing event occurs on the subsecond timescale and may result in activation of the opening of voltage-activated Na or Ca channels. The opening of said channels is often the event which initiates, and/or exacerbates, said increased frequency of depolarization. In ischemic and/or hypoxic situations, said increased frequency of depolarization is more likely to occur at inappropriate times and may have pathophysiological consequences (e.g., excessive release of glutamate to levels causing neuronal injury and death).

depolarization, increased duration of: a state of an excitable cell in which the length of time said cell is in the depolarized state is increased. Again, in ischemic and/or hypoxic situations, said increased duration of depolarization is more likely to occur at inappropriate times and may have pathophysiological consequences (e.g., excessive release of glutamate to levels causing neuronal injury and death).

depolarization, repetitive: depolarizing events that are discrete and recur over a period of time. Repetitive depolarization is a normal and beneficial phenomenon resulting, for example, in appropriately timed release of neurotransmitters from nerve terminals or rhythmic contractions of the heart. Pathophysiological situations may cause and/or be exacerbated by repetitive depolarizations that are inappropriately timed and/or are excessive in frequency, resulting, for example, in cardiac arrhythmias or excessive release of neurotrans-mitters. As employed to identify compounds of the invention, single cell electrophysiological methods are used to demonstrate the ability of repetitive depolarization to potentiate and/or accelerate block of ion channels by said compounds.

depolarization, sustained: depolarizing events that endure over time. As used herein, this term usually implies events that last for at least several seconds and may have

pathophysiological consequences (e.g., excessive release of glutamate to levels causing neuronal injury and death). As employed to identify compounds of the invention, rapid superfusion methods are used to demonstrate the ability of sustained depolarization to potentiate and/or accelerate block of Ca channels controlling glutamate release from brain nerve terminals said compounds.

hypoxic, hypoxia: levels of oxygen below those normally occurring in air, blood, or tissues. Sustained hypoxia may have pathophysiological consequences.

inhibition: reduction of the ability of a process to occur in the normal manner. For example, sustained depolarization or exposure to an ion-channel blocker may result in inhibition of the ability of a voltage-activated ion channel to conduct ions.

inhibition, preferential: circumstances favoring reduction of the ability of a process to occur in the normal manner. For example, compounds of the invention preferentially inhibit the ion-conducting ability of voltage-activated ion channels under circumstances of sustained or repetitive depolarization. This is due to the fact that said channels are more susceptible to block by compounds of the invention when said channels are in the conformational states that are more frequently generated by depolarization.

ion channels: protein molecules, embedded within and spanning the cell membrane, that are normally capable of conducting ions across the cell membrane through a transmembrane "pore" formed within said protein molecule.

ion channels, activated: an ion channel in a functional/conformational state that allows conduction of ions through its pore, across the cell membrane.

i n channels, activatable: an ion channel in a functional/conformational state that enables the channel to be activated by a particular initiating event. As applied to

voltage-activated ion channels, said initiating event is usually the depolarization of the cell membrane.

ion channels, inactivated: an ion channel in a functional/conformational state that prevents the conduction of ions through its pore, unless some event occurs that shifts it to the activatable functional/conformational state. As applied to voltage-activated ion channels, said event is usually the repolarization of the cell membrane: this is because depolarization of the cell membrane causes said channels to be activated only briefly and then revert to the inactivated state until the membrane is repolarized (i.e. returned to the normal, hyperpolarized value of membrane potential). Refer for background for additional discussion.

ischemia: local lack of blood supply in a tissue due to mechanical obstruction of blood vessels delivering blood to the tissue. For example, narrowing of the arteries (arterial stenosis) caused by cholesterol-generated formations (plaques) in the coronary arteries is a frequent cause of ischemia in the heart (myocardial ischemia). Another relevant example: blood clots occluding cerebral arteries is a frequent cause of brain ischemia, resulting in stroke. Compounds of the invention may be identified as neuroprotective agents in a stroke model, such as the rat middle cerebral artery occlusion ("MCAO") model employed herein (Example 9).

normally hyperpolarized cell: excitable cells in the "resting" state in healthy tissue normally exhibit membrane potential values, typically, of about -70 to -90 mV as measured electrophysiologically. This is termed a normally hyperpolarized cell. Notably, it is not "abnormal" per se for a cell to be found in a depolarized state, as depolarization is a process that initiates normal physiological processes such as neurotransmitter release and myocardial contractility. What is abnormal in the pathophysiological sense are cells undergoing excessive or inappropriate frequency or duration of depolarization, with pathophysiological consequences.

pathophysiol gical circumstances: situations producing adverse or undesirable bodily consequences, such as ischemic damage to the brain tissue in stroke or ischemic damage to the heart in myocardial infarction (heart attack).

Purkinje cell network of cardiac cells: a specialized, electrically coupled network of excitable cells intercalating the muscle of the ventricles of the heart. The purpose of this cell network is to rapidly conduct depolarizing impulses from the AV node of the heart throughout the "working" ventricular myocardium to ensure uniform and properly timed contraction of the ventricles to effectively pump blood. Ischemic damage to the Purkinje cell network can result in depolarizing impulses originating within the ischemic myocardium at "ectopic foci", producing irregular and inappropriate contractions of the heart which may be life threatening. Compounds of the invention should block these "ectopic beats" selectively, due to their ability to selectively block voltage-activated Na channels under circumstances of sustained or repetitive depolarization as occurs at ectopic foci originating within ischemic myocardium.

secretion: the release of substances from a cell or aggregate of cells (such aggregates of cells are found in secretory tissues such as the pituitary, pancreas, or adrenal medulla), for the purpose of influencing the behavior or functional state of other cells or tissues. Examples of secretory processes are the release of catecholamines from the adrenal medulla, release of growth hormone from the pituitary, and release of insulin from the pancreas. Ion channels comprising the targets of compounds of the invention, among them L-type voltage-activated Ca channels, play a central role in governing secretion. In most instances considered herein, secretion occurs via a process of **Ca-dependent exocytosis** of secreted substances that are packaged within small secretory granules; for example, catecholamines are concentrated in chromaffin granules within chromaffin cells, and catecholamines are released upon elevation of intracellular free Ca levels which is triggered by the opening of voltage-activated Ca channels.

SS1/SS2 ion-selectivity filter region: the "ion-selectivity filter site" is a site critical to the function of a broad range of voltage-activated ion channels, and has been well characterized for voltage-activated Na and Ca channels. Said site governs the ability of specific ionic species, e.g. Na or Ca, to pass through the pore of said channels. This site has recently been defined to comprise the "SS1/SS2" regions (also termed the "S5-S6" regions) in all known mammalian voltage-activated Na and Ca channels, which in turn comprise the therapeutically relevant targets of the actions of compounds of the invention. The amino acid sequences of the SS1/SS2 regions of a representative family of voltage-activated Na and Ca channels is provided in **Example 1**. Said channels possess four internally homologous repeat units, as illustrated in **Figure 3**. Each of the four repeat units contain an SS1/SS2 site, as defined in **Example 1** and further illustrated in **Figure 4A**. The structural arrangement of the pore formed by the juxtaposition of the SS1/SS2 regions of the four repeat units is illustrated in **Figure 4B**. Additional information on relevant properties of the SS1/SS2 region is also provided in the "Detailed Description" below.

SS1/SS2 ion-selectivity filter region, interaction with: certain compounds are known to block the conduction of ions through the pore of voltage-activated Na and Ca channels by binding to sites within the SS1/SS2 ion selectivity filter region. These include the toxins tetrodotoxin and saxitoxin, which selectively block said Na channels, and the cone snail venom peptides ω-conotoxin GVIA and ω-conotoxin MVIIA ("SNX111"), which selectively block said Ca channels. Compounds of the invention also block said channels by interacting with said ion selectivity filter, but in a use-dependent manner that, as detailed herein, makes them more attractive as therapies for disorders involving excessive or inappropriate activity of said channels (see definition of "use-dependent blocker").

therapeutically effective: as applied to the consequences of administration of a compound to an individual, able to produce significant alleviation of the pathophysiological consequences of the disorder or disease. Said alleviation may comprise symptomatic relief and or limitation of bodily damage resulting from said disorder or disease.

use-dependent blocker: as employed herein to describe a critically important property of compounds of the invention, a use-dependent blocker is a compound with the ability to block the activity of voltage-activated Na and/or Ca channels in the conformational states of said channels favored during sustained or repetive depolarization of the excitable cell membrane; such a compound is relatively less effective in its ability to block channels under hyperpolarizing conditions similar to those in quiescent ("normally hyperpolarized") excitable cells. The resulting therapeutic advantage is that compounds of the invention preferentially block voltage-activated Na and Ca channels and concomitantly the processes which they govern (e.g. neurotransmitter release) under pathophysiological circumstances producing sustained and/or repetitive depolarization of the cell membrane.

use-dependent blocker, "reverse-": an ion channel inhibitor is a "reverse-use-dependent blocker" if its channel blocking actions is alleviated, rather than enhanced, by repetitive and/or sustained depolarization. An example of this is the cone snail peptide w-conotoxin MVII-A ("SNX-111").

II. METHODS FOR IDENTIFICATION OF COMPOUNDS OF THE INVENTION:

A. Overview

The two properties of compounds of the invention which together define the scope of the invention are:

Property 1: Direct interaction with the SS1/SS2 ion-selectivity filter region of voltage-activated Na and/or Ca channels.

Property 2: Use-dependent block of voltage-activated Na and/or Ca channels.

The prior art has identified compounds that satisfy either property 1 or property 2. The invention claimed herein comprises the administration of compounds which satisfy both of the aforementioned properties. Compounds acting in this manner have not been previously identified or anticipated by the prior art, and constitute an improvement upon the prior art in terms of attractiveness as therapies for the indications described herein.

By blocking voltage-activated Na and Ca channels in a use-dependent manner through interaction with the SS1/SS2 ion-selectivity filter region of said channels, excessive cellular Ca entry and/or cellular hyperexcitability will be inhibited by compounds of invention, and the concomitant pathophysiological consequences will be attenuated. The aforementioned changes in the conformational states of said ion channels, which govern their corresponding functional states, correspond to finite changes in the shape and/or orientation of said ion channels which affect their ability to allow ions to move across the cell membrane through a transmembrane "pore" within the ion channel protein molecule [Triggle et al. (1989) Medicinal Res. Rev. 9, 123-180; Siegelbaum, S.A., and Koester, J. (1991) in Principles of Neural Science, 3rd Ed.,

Kandel, E.R. et al., eds., Norwalk CN, Appleton & Lange, pp. 66-79]. Translating important therapeutic goals into molecular terms, the object of this invention is to produce use-dependent compounds, administered as drugs, which selectively interact with said ion channels in the inactivated and/or the open state and result in attenuation of the ability of ions to move through said pore.

Below we provide herein generalized methods for establishing said two defining properties, of compounds of the invention:

B. **Methods for determining Property 1: Direct interaction with the SS1/SS2 ion-selectivity filter site of voltage-activated Na and/or Ca channels**:

Compounds of the invention directly bind to the "ion-selectivity filter site", a site critical to the function of a broad range of voltage-activated Na and Ca channels. This site has recently been defined to comprise the "SS1/SS2" region (also termed the "S5-S6" region) of 4 amino acid sequences in all voltage-activated ion channels claimed as therapeutically relevant targets of the actions of said compounds.

Property 1 may be established in either of two ways:

(a) The ability to competitively displace suitably labelled ligands which bind to the SS1/SS2 site at concentrations which approximate those required to demonstrate biological activity.

Ligands may be employed which are themselves be outside the scope of the current invention by mechanistic criteria (i.e. lack of use-dependence) but which bind to said site. An example described herein is the displacement of radiolabeled [^{3}H]-saxitoxin from binding to the SS1/SS2 site of voltage-activated Na channels (**Example 2 and Figure 5**). Other examples of such a ligand include but are not restricted to tetrodotoxin, which binds to said site on certain classes of voltage-activated Na channels [Satin, J., et al. (1992) Science **256**, 1202-1205], and the cone snail venom

peptides ω-conotoxin G-VI-A and ω-conotoxin M-VII-A, which bind to said site on certain classes of voltage-activated Ca channels [Boland, L., Morrill, J., and Bean, B. (1994) J. Neurosci., in press].

Regarding ω-conotoxin G-VI-A and ω-conotoxin M-VII-A ("SNX11"), they are compounds of the prior art which specifically block N-type Ca channels. They have been shown to compete at the ion-selectivity filter site of said channels with ions entering through the pore of the channels, among them Ca and Ba ions [Boland, L., Morrill, J., and Bean, B. (1994) J. Neurosci., in press]. However, in contrast to compounds of the invention, which exhibit use-dependent block of presynaptic Ca channels controlling glutamate release which is enhanced by depolarization of the nerve terminal membrane (as detailed *infra.*), block by said cone snail peptides of their Ca channel target, N-type Ca channels which are also a channel subclass involved in regulating neurotransmitter release [Bean, B.P. (1989) *Ann. Rev. Physiol.* **51**: 367-384] is antagonized or reversed by depolarization [Wurster, S., and Dooley, D.J. (1993) Abs., Soc. for Neurosci. 19, 1750].

Radiolabeled TTX and ω conotoxin G-VI-A are commercially available [Dupont/New England Nuclear, Boston MA], radiolabeled [^{125}I]ω conotoxin M-VII-A can and has been prepared [Valentino, K. et al. (1993) Proc. Nat. Acad. Sci. USA 90, 7894-7], and binding of both radiolabeled toxins to Ca channels of brain membranes has been described in the literature [Olivera, B.M. et al. (1987) Biochem. **26**, 2086-2090; Miljanich, G.P. et al. (1991) U.S. Patent #5,051,403]. Valid tests for competition of said toxins with prospective compounds of the invention for binding to the SS1/SS2 site of Na or Ca channels could be readily developed by one skilled in the art. Other means of labeling such compounds, e.g. labelling such a toxin or venom peptide with a fluorescent reagent such as dansyl chloride, are readily accessible methods which can be used by those of ordinary skill in the art.

Alternatively, compound to be tested could be subjected to competition for binding to the SS1/SS2 ion-selectivity filter region, with a radiolabeled compound previously shown to be a compound of the invention, for example CNS 1237 or any of the other substituted guanidine derivatives defined. Radioisotopes of hydrogen such as tritium (^3H) or carbon (^{14}C) can be readily incorporated into such substituted guandines during or after chemical synthesis by one of ordinary skill in the art.

(b) The ability to directly interact with residues within the structurally defined region of voltage-activated Na and/or Ca channels comprising said ion selectivity filter site.

As a result of analysis of the cloned genes of said Na and Ca channels, said site is known to constitute a set of 4 homologous amino acid sequences which are readily identifiable by one of ordinary skill in the art. The sequence information for the SS1/SS2 regions of particular subclasses of voltage-activated Na and Ca channels comprising representative targets for compounds of the invention is provided in **Example 1** *infra*..

The diagram of **Example 1** highlights the negatively-charged glutamate and aspartate residues shown by site-directed mutagenesis studies to be critically involved in regulating the tetrodotoxin and saxitoxin sensitivity of Na channels [Noda, M. et al. 19890 FEBS Lett. 259: 213-216; Terlau, H. et al. (1991) FEBS Lett. **293**, 93-96] and the ion selectivity of voltage-activated Na and Ca channels [Yang, J. et al. (1993) Nature **366**, 158-161; Kim, M.-K. et al. (1993) FEBS Lett. **318**, 145-148; for review, see Yellen, G. (1993) Nature **366**, 109-110]. A preferred embodiment of the invention is the subset of use-dependent compounds whose affinity, as shown by site-directed mutagenesis, is changed by the same site-directed mutations of said glutamate and aspartate residues within the SS1/SS2 regions which affect tetrodotoxin affinity and/or cation selectivity of said channels. An additional preferred embodiment is the subset of use-dependent compounds whose affinity, as shown by site-directed mutagenesis, is

altered by site-directed mutations of residues immediately to the right of said glutamate residues diagrammed in **Example 1**, as that locus, in addition, corresponds to the sites of additional mutations which alter tetrodotoxin's affinity for the ion-selectivity filter [c.f. Satin, J., et al. (1992) Science **256**, 1202-1205, and **Figure 6b**].

C. Methods for determining property 2: Use-dependence:

Compounds of the invention have the ability to block the activity of voltage-activated Na and/or Ca channels during sustained or repetitive depolarization of the excitable cell membrane, and are relatively less effective in their ability to block said channels under conditions found in quiescent, normally hyperpolarized excitable cells.

Property 2, may be established in either of two ways:

a) The use of electrophysiological methods to demonstrate the ability of repetitive or sustained depolarization to produce pronounced "use-dependent block" of voltage-activated ion channel targets.

This can be readily determined, for example, by the single cell patch clamp electrophysiological techniques readily accessible to one of ordinary skill in the art [Sakmann, B. and Neher, E. (1983) Single Channel Recording, New York, Plenum Press]. Such methods can be employed to demonstrate that repetitive or sustained depolarization enhances the potency of block of voltage-activated Na or Ca channels by prospective compounds of the invention, as examplified herein (c.f. **Example 2** and additional discussion below). Such methods may also be employed to demonstrate that prospective compounds of the invention accelerate the decay of the activity of voltage-activated Na or Ca channels upon repetitive or sustained depolarization (see **Example 3** and additional discussion below).

Variations and/or refinements of the electrophysiological methods described herein may also be employed, including but not restricted to: the use of single channel

recording, a variant of the patch clamp electrophysiology technique that affords information on the activity of individual ion channels [Sakmann, B. Nad Neher, E. (1983) Single Channel Recording, New York, Plenum Press]; and measurement of the electrical activity within brain slices *in vitro*, for example the use of hippocampal slice preparations [Horne, A. L., and Kemp, J.A. (1991) Br. J. Pharmacol. **103**, 1733-39].

b) The use of other methods, among them rapid kinetic techniques, to demonstrate ability of a compound to accelerate the decay of a biological process controlled by the voltage-sensitive ion channels blocked by said compound.

Exemplified herein (**Example 4**) and further described below is the ability of compounds of the invention to accelerate the decay of neurotransmitter release from brain nerve terminals, measured *in vitro* using rapid kinetic techniques [Goldin S. M. (1990) U.S. Patent #4,891,185]. Said acceleration of decay results from use-dependent block of presynaptic Ca channels which govern neurotransmitter release from brain nerve terminals. Other examples of the acceleration of the decay of processes controlled by voltage-activated ion channels include the acceleration of the decay of the membrane depolarizations responsible for muscle contraction, a process also governed by voltage-activated ion channels [Cannon, S. C. et al. (1992), *ibid.*].

Additional methods, and/or variations and refinements of the methods described herein may be employed to measure neurotransmitter release or the activity of voltage-activated Na or Ca channels governing said release, among them measurement of the electrical activity induced by neurotransmitter release within hippocampal slices, fluorescence and/or enzymatic methods to analyze synaptosomal neurotransmitter release, and stopped-flow rapid kinetics [Cash, D.J., and Katragadda, S. (1987) Biochem. **26**, 7556-7570; Karpen, J.W. et al. (1983) Anal. Biochem. **135**, 83-94].

General considerations for design of methods to identify use-dependent block: frequency-dependent channel block, channel block induced by sustained depolarization

Some therapeutic applications may be more likely to be undertaken by compounds acting preferentially to block a voltage-activated channel comprising a relevant therapeutic target when said target is experiencing high stimulus frequencies. This would be a desirable property, for example, of an anticonvulsant agent, as epilepsy is known to involve rapid bursts of high frequency firing of action potentials at epileptic foci. A second category of therapeutic applications, such as limitation of ischemic brain damage, may be more appropriately treated by a compound which also exhibits frequency-dependent block of voltage-activated ion channels when the frequency of stimulation is relatively low. Accordingly the examples presented here are not intended to limit the scope of the compounds falling within the claimed invention: certain compounds may exhibit marked frequency dependence, for example, only when the stimulus frequency exceeds 30 Hz, but may nonetheless have therapeutic advantages, perhaps superior to those of the compounds of the invention which serve as examples herein.

Still a third category of therapeutic applications may benefit from compounds which act to block channel activity induced by sustained depolarization of the cell membrane. It has been demonstrated that in sustained brain ischemia, the depletion of cellular ATP levels [Shimizu, H. et al. (1993) Brain Res. **605**, 33-42] causes dissipation of the Na and K gradients and concomitant sustained depolarization of the cell membrane [Zivin, J.A. and Choi, D.W. (July, 1991) Sci. Amer. **265**, 36-43]. Said sustained depolarization generates hyperactivity of neuronal ion channels [Alzheimer, C. (1993) J. Neurosci. **13**, 660-673].

Thus, although the aforementioned examples and test protocols, described exemplified herein, constitute a straightforward and convenient way which enables one

already skilled in the art of electrophysiological and/or rapid kinetic methods to identify a compound of the invention, they do not limit the development, by one skilled in the art, of other tests to demonstrate use-dependent block of said ion channels by compounds whose properties fall within the scope of the invention as defined and illustrated herein. Said electrophysiological and/or rapid kinetic methods as well as other approaches can be further refined and developed. They can and will be used to identify additional compounds satisfying one of two the key criteria comprising the invention: use-dependence in the ability of the compound to block an ion channel target of the invention.

III. DETAILED DESCRIPTION OF SOME SPECIFIC METHODS FOR IDENTIFICATION OF COMPOUNDS OF THE INVENTION:

A. Specific methods for determining Property 1: Direct interaction with the SS1/SS2 ion-selectivity filter site of voltage-activated Na and Ca channels.

1. Overview of the structure and function of the SS1/SS2 ion-selectivity filter site derived from molecular biology and biophysics

To convey a full appreciation of the rationale for the specific methodology employed for identification of compounds of the invention, it is necessary to review some salient features of the structure of said ion channels as recently resolved by basic research in molecular biology and channel biophysics.

Hille and others [Hille, B. (1975) Biophys. J. **15**, 615-619] have hypothesized, based on mechanistic and structure-activity studies, that guanidine-based ion-channel blockers such as tetrodotoxin act to block the "ion-selectivity filter" region of the channel. The ion-selectivity filter limits the free diffusion of ions through its transmembrane pore. The size and structure of this critical region defines the permeability of a given ion through the channel, based on its size and ionic radius [Hille, B. (1984) Ionic Channels of Excitable Membranes, Sunderland MA, Sinauaer 226-303]. The positively charged guanidinium group may interact with negatively charged residues in the filter, and high affinity block is imparted by interaction of other groups on that toxin with adjacent residues.

The gene for the tetrodotoxin-sensitive subclass of Na channels has been cloned, and the channel has been functionally expressed in *in vitro* preparations such as frog oocytes that enable direct electrophysiological measurement of the functional properties of said channels. Hille's hypothesis was rigorously tested and confirmed based on

recent results stemming from the functional analysis and site-directed mutagenesis of cloned, expressed Na channels: as illustrated in **Figure 6b**, a single point mutation in the SS1-SS2 region of cardiac Na channels, which are TTX-insensitive, can convert them into TTX-sensitive channels closely resembling native *neuronal* Na channels in their pharmacological properties Satin, J., et al. (1992) Science **256**, 1202-1205].

It is clear that there is a high degree of structural and functional similarity between Na and Ca channels (**Figure 3**). This similarity extends to the SS1-SS2 ion-selectivity filter region of these voltage-activated channels, as recently reviewed by Heinemann et al. [Heinemann, S. H., H. Terlau, W. Stuhmer, K. Imoto, S. Numa (1992) Nature **356**, 441-443]. A dramatic demonstration of this is the fact that a single mutation in this region, the SS1-SS2 site (see **Figure 6**) can change the ion-selectivity characteristics of rat brain Na channels to those of Ca channels [Heinemann, S. H. (1992), *ibid.*].

Specifically, **Figure 6a** displays sequence data for the SS1/SS2 region of internal repeat I of a variety of Ca and Na channel clones from both rat and human cDNAs. The negatively charged residue, glu (E) or asp (D), are the highly conserved amino acids hypothesized, in TTX-senstitive Na channels (e.g., brain I - III) to be the site of interaction of the positively charged guanidinium group of TTX with the selectivity filter. The adjacent hydrophobic residue (tyr [Y] or phe [F]) confers TTX-sensitivity, as very recently reported by the independent studies of two different laboratories [Satin, J., et al. (1992) Science **256**, 1202-1205; Backx, P.H. et al. (1992) Science **257**, 249-251]. **Figure 6b** illustrates repeat I of a generalized voltage-sensitive Na channel (85). The seven amino acid SS2 sequence is shown, and the differences between the TTX-resistant ("RHII") and TTX -sensitive (I -Brain II) isoforms are indicated.

Figure 4 displays salient structural information on voltage-activated Ca and Na channels. Panel A is a Diagram of sodium channel transmembrane topology adapted from a description provided by Noda, M. et al. [(1986) Nature **320**, 188-192.]. The homologous internal repeats I - IV each contain six membrane spanning segments (S1 - S6) represented as cylinders. The SS1/SS2 ion selectivity filter region is represented by the separation between S5 and S6 in each of the four internal repeats.

A structural model has been proposed based on evidence that the SS1/SS2 region of each of four internally homologous repeat units within the channel line the mouth of the pore, and that the ion-selectivity filter site is defined by the juxtaposition of amino acid residues of SS1/SS2 sites from each of the four internal repeat units. This is represented in Panel B of Figure 4 [Adapted from Salkoff et al. (1987) TINS **10**, 523], which represents the arrangement of said transmembrane segments of Panel A as viewed in the direction perpendicular to the membrane. This structural model was first proposed for Na channels by Guy and Seetharamulu [Guy, H.R. and Seetharamulu, P. (1986) Proc. Nat. Acad. Sci. USA **83**: 508-512]. A critical feature of said model, the juxtaposition of the SS1/SS2 sites of each of the four internal repeat units forms the ion selectivity filter site, has been confirmed by the fact that single mutations within each of the four SS1/SS2 sites of voltage-activated sodium channels alter ion selectivity and/or tetrodotoxin affinity [Noda, M. et al. 19890 FEBS Lett. 259: 213-216; Terlau, H. et al. (1991) FEBS Lett. **293**, 93-96].

Recent site-directed mutagenesis studies of voltage-activated Ca channels have convincingly shown that the model depicted in **Figure 4a and 4b** also comprise a valid representation of the molecular structure of the ion-selectivity filter of Ca channels as well as Na channels. All known voltage-activated Ca channels have negatively charged glutamate residues in each of the putative pore-lining SS1/SS2 regions. As an example of this, **Figure 4c** [adapted from Yang, J. et al. (1993) Nature **366**] displays amino acid sequence information from the SS1/SS2 region of each of the four internal repeats of

cardiac L-type Ca channels, and highlights the negatively charged glutamate residues "E" in the SS2 region of each repeat. Two research groups demonstrated that replacement of any one of these residues, by site-directed mutagenesis, substantively alters the selectivity of the pore of said Ca channels for conduction of monovalent and divalent cations [Yang, J. et al. (1993) Nature **366,** 158-161; Kim, M.-K. et al. (1993) FEBS Lett. **318**, 145-148]. These results solidly confirm the view that the structural basis of Ca selectivity resides to a large extent in this ring of four negatively charged residues lining the pore, thus proving that each of the four internally homologous SS1/SS2 regions contribute critical structural features to the ion-selectivity filter common to said Ca as well as Na channels. Further systematic application of site-directed mutagenesis is providing a detailed picture of the asymmetric arrangement of the four SS1/SS2 sites lining the pore, with particular attention paid to the aforementioned four glutamate residues.

As will be expanded upon herein, compounds of the invention synthesized and identified to date are all substituted guanidines, as are tetrodotoxin and saxitoxin. Further clarification of the molecular interaction between compounds of the invention should thus be achievable by employing site-directed mutagenesis studies targeted to the SS1/SS2 site, in analogy to the aforementioned studies recently performed to clarify the interactions of tetrodotoxin and small cations with said site.

The sequence similarity in the SS1/SS2 site includes human as well as rat Ca and Na channel cDNA's (refer to sequence information in **Example 1**). This information is of immense practical therapeutic utility, because drug candidates developed based on biological feedback from *in vitro* assays employing material derived from rats (e.g. rat brain nerve terminal preparations employed in examples 4 and 6 *infra.*) and *in vivo* models of disease states (e.g. the rat kainate neurotoxicity model and the rat stroke model, examples 8 and 9 infra) are expected to exert similar actions in humans because the sites to which the drug candidates bind are structurally

very similar if not identical. This consideration has strongly influenced our decision to diligently pursue the creation of use-dependent blockers of the SS1/SS2 binding site of voltage-activated Na and Ca channels.

2. Creation of a compound series which interacts with the SS1/SS2 ion selectivity filter site.

Certain novel acenaphthylguanidines, among them N,N'-Bis (5-acenaphthyl) guanidine (ref. our prior patent application), initially received our attention as compounds deemed likely to interact with the ion-selectivity filter region of voltage-activated channels. Regarding the molecular basis of the channel block exhibited by compounds of the invention, we considered the structural properties of said compounds in aqueous solution: What conformation might such a compound be in when it first encounters its ion-channel target? NMR studies of the conformation in aqueous solution of these compounds, for example that of N, N'-diacenaphthylguanidine, suggested that Π–Π stacking of the two planar acenaphthyl groups forced the positively charged guanidinium group to extend away from the molecule and create a structure whose size and shape roughly resembles TTX. This is illustrated for the case of two compounds of the invention, with the aid of the computer-generated molecular models as shown in **Figure 7**. This result was supported by the results of molecular modelling/energy minimization studies, which indicated that a "stacked conformation" of these compounds was energetically accessible in aqueous solution.

The aforementioned chemical considerations do not at present comprise a rigorous theoretical framework for the chemical basis of the interaction of compounds of invention with the SS1/SS2 site occupied by tetrodotoxin. Nonetheless these observations, together with the dual actions of such compounds on voltage-activated Na and Ca channels (see below), first led us to the formulation and validation of the following hypothesis, critical to the current invention: the aforementioned acenaphthyl guanidine derivatives interact with the ion-selectivity filter (SS1/SS2) binding site

shared by voltage-activated Na and Ca channels and known to be the site of action of tetrodoxin's and saxitoxin's blockade of the type I and II neuronal Na channel subclasses

Methods for generating use-dependent block at the ion-selectivity filter site of voltage-activated ion channels utilize the administration of particular substituted guanidines or analogs thereof. Said substituted guanidines contain a positively charged guanidinium group which has been shown to interact with a negatively charged amino acid residue or residues within the ion-selectivity filter site. Analogs of said substituted guanidines comprise compounds whose structural properties also predispose them to interact with the ion-selectivity filter site in a use-dependent manner. Said analogs could include any compounds that contain groups that are likely to exist as a positively-charged ionic species under the conditions likely to pertain within voltage-activated ion channels. Derivatives of the ammonium cation form a most numerous and diverse group of analogs of the guanidines. Said analogs include, but are not limited to, substituted amidines, for example formamidines, substituted hydroxylamines, and substituted hydrazines. It is additionally pertinent with respect to the aforementioned chemical classes of compounds that there is evidence that the formamidinium cation, the hydroxylammonium cation, etc. interact with a negatively charged residue or residues within the ion-selectivity filter site (Hille, B., J. Gen Physiol. 58, 599-619; reviewed in Hille, B (1992) Ionic Channels of Excitable Membranes, 2nd Edition, Sunderland, MA., Sinauer Assoc.]. It is possible that compounds that do not contain a full positive charge, nevertheless may mimic this interaction by means of other groups within the molecule that may utilize non-ionic forces, e.g. hydrogen bonding or dipole-dipole interactions.

By attaching suitable chemical substituents to the positively charged guanidinium moiety or analog thereof, we demonstrate herein the creation of use-dependent blockers of the ion-selectivity filter site of voltage-activated ion channels.

Said substituents include but are not limited to acenaphthylenes, halogenophenyls, fluorenes, para- or meta-butylphenyls, para-terbutylbenzyls, anthracenes, and methoxynaphthylenes.

3. Compounds of the invention which competitively displace [^{3}H]-saxitoxin from the SS1/SS2 ion-selectivity filter site of voltage-activated Na channels

The specific method exemplified herein demonstrates herein that compounds of the current invention are capable of competitively displacing saxitoxin from the SS1-SS2 (ion-selectivity filter) binding site within voltage-activated neuronal type II Na channels (**Example 5**). This interaction occurs at concentrations at which compounds of the invention demonstrably block voltage activated Na channels (**Examples 2 and 3**) and Ca channels (**Examples 6 and 7**).

Using the radiolabeled TTX analogue [^{3}H]saxitoxin, said acenaphthyl guanidine derivatives were directly shown to interact with the TTX binding site on the ion-selectivity filter region of neuronal Na channels. As demonstrated in **Example 5** and **Figure 5**, [^{3}H]STX binding was measured in rat brain synaptosomes essentially as previously described [Gusovsky, F. et al., (1990) Brain Res. **518**: 101-106]. The K_d for [^{3}H]STX binding to the synaptosome preparation was found to be about 2.5 nM. TTX inhibited the [^{3}H]STX binding with a K_i of about 25 nM. As shown, said guanidine derivatives provided as examples of compounds of the invention, at concentrations ranging from ~1 to 30 uM, were found to increase the K_d of [^{3}H]STX binding, with no significant change in the [^{3}H]STX binding site density, indicating a purely competitive mode of inhibition.

B. Specific methods for establishing that compounds of the invention block voltage-activated Na and/or Ca channels

Before resorting to more involved mechanistic studies required to establish use-dependent block of voltage-activated Na and/or Ca channels, it is desirable from the standpoint of cost and efficiency to employ relatively more simple and rapid methods to determine whether, and at what concentrations, candidate compounds block said channels.

The existence of a cell line expressing the same subclass of TTX and STX-senstitive Na channels found in brain axons and nerve terminals (Type II), afforded the opportunity to demonstrates the efficacy of compounds of the invention in blocking said Na channels. **Example 8**, which entails the use of a rapid and convenient radioisotopic flux assay, demonstrates the ability of certain compounds of the invention to block said voltage-activated Na channels.

The known structural homologies between voltage-activated Ca and Na channels and the therapeutic utility of blockers of Na channels (see "Background") led us to determine the ability of compounds of the invention to block voltage-activated Ca channels in addition to said Na channels. **Examples 6 and 7** confirm this expectation, and demonstrates a rapid and convenient methodology using depolarization-stimulated 45Ca entry into, receptively, brain nerve terminal preparations (synaptosomes) through presynaptic Ca channels and into clonal GH4C1 pituitary cells through L-type Ca channels.

Said examples further establish that a subset of compounds of the invention have dual actions on two subclasses of Ca channels, based on direct measurement of the ability of compounds of the invention to block ^{45}Ca entry through said channels. **Example 6** demonstrates this for voltage-activated (P- and Q- type) Ca channels which

. control neurotransmitter release from brain nerve terminals. **Example 7** demonstrates this for L-type Ca channels, the channel subclass found in cardiovascular cells, cells of skeletal muscle, and secretory cells including those of the pituitary and the adrenal medulla.

A subset of compounds of the invention block both voltage-activated Na channels and Ca channels. In several cases illustrated herein, compounds of the invention block said Ca channels at concentrations comparable, on an order of magnitude basis, to those which block voltage-activated Type II Na channels (**c.f Examples 6, 7 and 8**). This further establishes that a subset of compounds of the invention possess a broad spectrum of action on voltage-activated Na and Ca channels by virtue of their interaction with the ion-selectivity filter (SS1/SS2). Said broad spectrum of action is a desirable property for neuroprotective agents [Kucharczyk, J. et al. (1991) Radiology **179**, 221-227], and should also be a desirable property for cardioprotective agents as well.

C. Specific methods for establishing Property 2: Use- dependent block of voltage-activated Na and Ca channels and processes which they govern:

In contrast to the actions of tetrodotoxin and saxitioxin at the SS1/SS2 ion selectivity filter binding site, compounds of the invention exhibit pronounced use-dependence in their ability to block said Na channels, as reflected by the ability of depolarization of the cell membrane to enhance their channel blocking ability (**Examples 2 and 3**). Furthermore, in contrast to certain cone snail peptides hypothesized to interact with the ion-selectivity filter of voltage-activated Ca channels [Boland, L., Morrill, J., and Bean, B., J.Neurosci., in press, 1994], compounds of the invention exhibit use-dependence in their ability to block said Ca channels as reflected in the ability of depolarization of nerve terminals to enhance their channel-blocking ability (**Example 4 and Figure 8**). In essence, our novel strategy has resulted in the

creation and identification of the only compounds which both interact with the SS1/SS2 binding site and are known to exhibit use-dependent blockade, as operationally defined herein, of said voltage-activated Na and Ca channels containing said structurally homologous binding site.

1. Specific methods for electrophysiological determination of use-dependent block of voltage-activated Na channels.

The aforementioned CHO cell line expressing Type II Na channels can be employed to assess the actions of compounds of the invention on said Na channels, directly demonstrating the use-dependent mechanism of action of compounds of the invention on voltage-activated ion channels constituting a therapeutically desirable target for drug action. The actions of these compounds on sodium channels were examined using the whole-cell voltage clamp recording technique. As illustrated (**Figure 9a**), CNS 1237 at low micromolar concentrations exhibits a high degree of depolarization-dependent block during stimulation at higher frequencies. These effects are similar to the actions of a number of local anesthetic, anticonvulsant and antiarrhythmic drugs on cardiac sodium channels [Hille, B. (1984) Ionic Channels of Excitable Membranes, Sunderland MA, Sinauaer 226-303]. While not wishing to be bound by theory, we hypothesize that CNS 1237 interacts preferentially with open and/or inactivated states of the channel, relative to its actions on channels in the closed but activatable state favored in resting, hyperpolarized cells.

As shown by **Examples 2 and 3,** compounds of the invention may be identified by rigorous examination of their interaction with voltage-activated ion channels using single-cell electrophysiological techniques. Below are described the specific criteria that may be used to identify compounds acting as use-dependent blockers of a specific voltage-activated ion channel.

a. Repetitive channel activation enhances the potency of compounds of the invention for blocking said voltage-activated ion channel.

Figure 9a demonstrates the ability of one of several compounds of the invention, CNS 1237, to block voltage-activated Type II neuronal Na channels in a frequency-dependent manner. This characteristic is contrasted with the relative inability of one of the compounds of the prior art, tetrodotoxin, to produce the same phenomenon when tested as described (**Figure 9b**). The experimental protocols employed are detailed in **Example 2**. As illustrated, CNS 1237 is markedly more potent in its ability to block type II Na channels when the channels are repetitively opened by trains of depolarizing impulses. The IC50 for tonic blockade of this channel is ~100 uM, whereas the IC50 for additional frequency-dependent blockade of this channel is about tenfold greater. In contrast, tetrodotoxin exhibits potent block of said Na channels irrespective of whether the channels are repetitively opened by trains of depolarization of the cell membrane containing said channel (**Figure 9b**).

b. Repetitive channel activation accelerates the rate at which compounds of the invention block said voltage-activated ion channels.

The aforementioned Example entails stimulation protocols which demonstrate that CNS 1237 and other compounds of the invention achieve a significant degree of frequency-dependent block during merely several seconds of high-frequency stimulation. While compounds such as CNS 1237 may be particularly advantageous for certain therapeutic indications, compounds of the invention, at optimal therapeutic concentrations in mammals, may require minutes or even hours of repetitive stimulation in order for them to produce frequency-dependent block of a voltage-activated ion channel targets of the invention. Such a relatively slow onset of block at therapeutic concentrations may be tolerable and even desirable, particularly for therapy of chronic indications such as LAS [Appel, S.H. (1993) Trends Neurosci. **16**, 3-5].

Even in the case of acute treatment to limit neuronal damage resulting from neuronal damage resulting from several minutes of transient global ischemia, treatment with compounds of the invention may be delayed for at least a few hours, nonetheless producing a significant degree of neuroprotection.

In certain cases involving particular compounds of the invention, it may not be practical to generate complete dose-response curves for tonic blockade of an ion-channel target of the invention. For example, solubility may limit the ability to test certain compounds at concentrations producing a high degree (>50%) of tonic block of said ion-channel target. In addition, the time course of frequency-dependent block may be relatively slower and/or the maximal frequency of stimulation of repetitive action potentials mediated by said ion channels may be relatively lower. These practical considerations may limit the ability of the experimenter to generate a complete set of data such as that provided for CNS 1237 in **Example 6**.

Fortunately, it is not essential to resort to protocols as involved or elaborate as those cited in (a) above, in order to clearly demonstrate frequency-dependent block of an ion channel target of the invention. A simplified experiment, such as that illustrated in **Figure 10** and described in **Example 3**, may be employed to demonstrate frequency-dependent block of an ion channel of the invention without the need to generate a complete dose-response curve employing a variety of concentrations compound to be tested. **Figure 10 and 10b** demonstrates the ability of repetitive nerve stimulation to accelerate the rate at which several of the compounds of the invention block a voltage-activated ion channel of the invention, the Type II neuronal sodium channel subclass of said channels, when the cloned gene for the α -subunit of said channel is expressed in a CHO cell line. In this example, compounds of the invention CNS 1237 and CNS 5149 , are subjected to depolarizing stimuli at a relatively low frequency: 0.1 Hz, in contrast with the higher frequencies previously illustrated in **Figure 9** and **Example 2**. When said CHO cells are preincubated with said compounds of the invention, at

concentrations sufficient to produce an appreciable but less then total block of Na current (in this case ~40%), said low frequency of stimulation produces an additional component of blockade of Na current, due either to enhancement of the rate of onset of block or increase in the potency of the compound's ability to block said Na channels. when equilibrium between the blocked and unblocked states of the channel at this stimulus frequency has been reached.

This characteristic is contrasted with the inability of a compound of the prior art, tetrodotoxin, to produce the same phenomenon when tested as described: as shown, at concentrations of tetrodotoxin (50 nM) sufficient to achieve ~40% tonic block, low levels of stimulation do not accelerate or potentiate additional block. As illustrated in **Figure 10c**, in contrast to said compounds of the invention, tetrodotoxin rapidly blocks these said Na channels, in a manner largely independent of repetitive stimulation.

These two independent criteria, exemplified above, demonstrate that compounds of the invention may be identified by electrophysiological protocols involving high-frequency stimulation, and/or alternatively by methods involving low-frequency stimulation. The specific stimulation frequencies employed in **Examples 2 and 3** are merely illustrative: some compounds, exemplified by CNS 1237, may exhibit frequency-dependent behavior in both experimental situations, and their specific kinetic properties may afford the practical opportunity to test them by both methods. Alternatively, it may be more convenient or practical to subject certain other compounds to only one of the two tests to determine whether said frequency- and/or depolarization dependence of channel block falls within the scope of the definition of said compound of the invention. This may be desirable, for example, if a compound to be tested is more pronounced in its ability to block a voltage-activated ion channel when it is stimulated at a particular rate or a particular frequency.

2. Specific methods for identification of c mpounds of the invention which exhibit use-dependent bl ckade f neurotransmitter release g verned by voltage-activated Ca channels.

A preferred embodiment of the current invention is the use of compounds of the invention to block, in a use-dependent manner, the voltage-activated Na and/or Ca channels which regulate neurotransmitter release, in particular glutamate release [McBurney et al. (1992) *J. Neurotrauma* **9**, Suppl. **2**, S531-S543; Katragadda, S., et al., (1992) Abs. Soc. for NeuroSci. Abs. **18**, 436]. We have already demonstrated the ability of compounds of the invention to exhibit use-dependent block type II Na channels, a Na channel subclass governing neurotransmitter release. Furthermore, as detailed in **Example 6** and discussed above, we have demonstrated the ability of compounds of the invention to block the presynaptic Ca channels governing neurotransmitter release from brain nerve terminals. Below, we demonstrate use-dependence in the ability of compounds of the invention to block the Ca channels governing glutamate release, by a rapid superfusion method that can be readily employed to demonstrate use-dependent blockade of Ca-channel mediated release of other neurotransmitters as well.

We have adapted a novel rapid superfusion system [Goldin, S. M., U.S. Patent #4,891,185] for the purpose of measuring ^{3}H-glutamate release from brain nerve terminals. The method involves first preloading rat brain synaptosomes with ^{3}H-glutamate via the Na-dependent glutamate uptake system. The preloaded nerve terminals are retained in a superfusion chamber accessed by high-speed, solenoid-driven valves. Microcomputer-operated circuitry controls the timing of valve operation; the valves control the delivery under nitrogen pressure of depolarizing pulses of high K^+ buffer, Ca, and/or drugs to the synaptosomes. The ^{3}H-glutamate-containing effluent is continuously collected in a high speed fraction collector on a timescale as short as 30 msec. The high solution flow rate and minimal dead volume of the superfusion

chamber, afford rapid solution changes and precise control of the chemical microenvironment of the nerve terminal preparation.

Compounds of the invention, notably N, N'(di-acenaphthyl)-guanidine and analogues thereof, exhibited use-dependent block of ^{3}H-glutamate release. Characterization of the kinetics of blockade of glutamate release reveals this property of use-dependence, and provide a rigorous test to define this property of compounds of the invention. As described in **Example 4** and **Figure 8a**, the decay of the persistent component of glutamate release can be fitted by a single exponential with a decay time constant of about one to several seconds. As further illustrated therein, N'-acenaphthyl-N'-methoxynaphtyl guanidine inhibits the persistent component of glutamate release and accelerates the decay of the Ca-dependent release event (**Figure 8a and 8b**); this is closely analogous to the "classical" observation of the acceleration of decay of action potentials by use-dependent ion channel antagonists such as lidocaine [Bean, B. P., C. J. Cohen and R. W. Tsien (1983) J. Gen. Physiol. **81**, 613-642], and constituted the first line of evidence that this acceleration of decay was due to depolarization-dependent block of the presynaptic Ca channels controlling Ca-dependent glutamate release. Our novel approach to drug discovery provided the feedback to our medicinal chemistry effort which has resulted in the creation of a variety of additional compounds of the invention exhibiting said operationally defined use-dependence [See **Example 4 and Figure 8b**].

Said use-dependent block is a therapeutically attractive property for an antiischemic agent, as such an agent would be more effective in blocking persistent, excessive glutamate release activity observed in ischemia than the brief, transient responses characteristic of "normal" release events. For example, CNS 1237 (acenaphthyl-methoxynaphthyl-guanidine), at [1 M], has little effect on the initial amplitude of the glutamate release event, but after 1-2 sec of sustained depolarization will reduce the rate of release by ~40-60%. This sustained glutamate release *in vitro*

should be a realistic reflection of *in vivo* pathophysiology: trains of fast Na action potentials invading release sites should produce a maintained depolarization if the frequency is >10Hz, because N- and P-type Ca channels inactivate more slowly than action potential Na channels, on a timescale of 30-100 msec rather than ~1-2 msec (24,91).

Further evidence convincingly demonstrates that said depolarization-dependent block of ^{3}H-glutamate release from brain nerve terminals by compounds of the invention is due to blockade of presynaptic Ca channels. Studies of glutamate release as measured by rapid superfusion have demonstrated [Turner, T. J., M. E. Adams and K. Dunlap (1992) Science **258**, 310-313; Turner, T. J., M. E. Adams and K. Dunlap (1993) Proc. Nat Acad. Sci. USA **90**, 9518-22; Katragadda et el. (1993) Soc. for Neurosci. Abs. **19**, 1750] that the aforementioned Ca channel selective spider venom peptide ω-aga-IVA blocks glutamate release from rat brain synaptosomes, establishing that the P- and/or Q-type type Ca channel(s) constitute the presynaptic Ca channel subclass(es) controlling the Ca-dependent glutamate release which is blocked by our novel compound series. This is further illustrated in **Example 4**, which utilizes the rapid superfusion method used herein to identify compounds of the invention which block glutamate release [Goldin, S.M. (1990), U.S. Patent #4,891,185].

We have also demonstrated that, as independently reported [Pocock, J.M., Venema, V., and Adams, M. (1992) Neurochem. Int. **20**:263-270], ω-aga-IVA and ω-conotoxin M-VII-C block the uptake of radioisotopically labeled Ca (^{45}Ca) into synaptosomes (**Example 6**), establishing additional direct evidence that said P-type and/or Q-type Ca channels are found presynaptically and control the release of glutamate and, presumably, other neurotransmitters from brain nerve terminals. Notably, compounds of the invention which exhibit depolarization-dependent block of ^{3}H-glutamate release from brain nerve terminal preparations also block the uptake of

^{45}Ca into synaptosomes prepared by the same method used to study ^{3}H-glutamate release from brain nerve terminals (**Example 4**).

The aforementioned results, taken together, indicate that depolarization-dependent block of presynaptic Ca channels is the mechanism responsible for the depolarization-dependence of ^{3}H-glutamate release from brain nerve terminal preparations by compounds of the invention.

It is further known that, in contrast to the aforementioned compounds of the invention, prolonged or repetitive depolarization relieves rather than potentiates block of voltage-activated Ca channels by ω-aga-IVA and ω-conotoxin M-VII-C [Mintz, I.M., and Bean, B.P. (1993) Abs. Soc. Neurosci. 19, 1478; Turner, J. T. et al (1993) Proc. Nat Acad. Sci. USA, 9518-9522....]. This has also been shown to be the case for structurally related cone snail peptides ω-conotoxin M-VIIA ("SNX-111') and ω-conotoxin G-VI-A [Wurster, S., and Dooley, D.J. (1993) Abs., Soc. for Neurosci. **19**, 1750; Turner, J. T. et al. (1993) Proc. Nat Acad. Sci. USA **90**, 9518-9522]. Our rapid superfusion measurements of the effects of ω-aga-IVA and ω-conotoxin M-VII-C on glutamate release confirm this observation: as described in **Example 4**, and **Figure 8b**, the decay constant for Ca-dependent neurotransmitter release blocked by said venom peptides is prolonged rather than reduced, in direct contrast to the actions of compounds of the invention on Ca-dependent ^{3}H-glutamate release from brain nerve terminal preparations. **Figure 11** further explores this phenomenon as regards the actions of ω-contotoxin MVII-C. Said toxin potently blocks the early kinetic ("phasic") component of glutamate release (measured during the first 600 msec of depolarization) , elicited by depolarization with 25 mM K, with much greater potency than displayed in its ability to block the "persistent" component (i.e., release measured during the time interval from 0.9 to 5.0 sec). The toxin exhibits biphasic block of the phasic component, with an IC_{50} of ~0.1 μM for block of the high affinity component. The IC_{50} for block of the persistent component was ~1μM.

Methods very similar to those described in **Example 4** and illustrated in **Figure 6b** and **Figure 11** have been employed to measure the Ca-dependent, depolarization-stimulated release of other neurotransmitters, among them GABA [Turner, T.J. and Goldin, S.M. (1989) *Biochem.* **28**, 586-593], norepinephrine [Turner, T.J. and Goldin, S.M. (1985) *J. Neurosci.* 5, 841-849], dopamine [Katragadda, S., Brown, M.L., Turner, T.J., and S.M. Goldin (1990) *Abs. Soc. for Neurosci.* **16**, 143; Turner, T. J., M. E. Adams and K. Dunlap (1993) *Proc. Nat Acad. Sci. USA* 90, 9518-22], and acetylcholine [Pearce, L.B. et al. (1991) *J. Neurochem.* **57**, 636-647]. Compounds of our own prior art which are substituted guanidines related to compounds of the current invention, and which block glutamate release (Goldin, S.M. et al. PCT/US92/01050) have also been shown to inhibit the release of other transmitters at roughly similar concentrations (data not illustrated). Accordingly, it is our firm expectation based on the above and the results described in **Example 4, Figure 8b** and **Figure 11** that a large subset of compounds of the invention will be useful in inhibiting release of the aforementioned neurotransmitters and other neurotransmitters as well.

IV. INDICATIONS FOR THERAPEUTIC USE OF COMPOUNDS OF THE INVENTION.

A. Overview: Voltage-activated ion channels comprising the molecular targets of compounds of the invention

The current invention as defined in the present application identifies the binding site as the ion-selectivity filter/SS1-SS2 regions of the voltage-activated Na and/or Ca channels blocked by compounds of the invention. The structural homology between voltage-activated Na channels and voltage-activated Ca channels and subclasses thereof [See figure 3 and Catterall, W.A. (1988) *Science* **242**, 50-52; Heinemann, S. H., H. Terlau, W. Stuhmer, K. Imoto, S. Numa (1992) *Nature* **356**, 441-443; Backx et al., *Science* 257 (1992) 248-251; Zhang, J.-F. et al. (1993) *Neuropharmacol.* **32**, 1075-1088; Snutch, T.P. and Reiner, P.B. (1992) *Curr. Opin. Neurobiol.* **2**, 247-253], is the explanation for the fact that a subset of the compounds of the current invention have channel blocking actions on multiple subclasses of said Na channels and Ca channels.

A preferred embodiment of the current invention is the use-dependent blockade of disorders involving excessive and/or inappropriate activity of neuronal voltage-activated Na and/or Ca channels. Accordingly such compounds of the invention will be useful for the treatment of a variety of disorders involving inappropriate or excessive activity of said ion channels, including disorders of the nervous system such as produced by a hypoxic/ischemic insult to the brain or in epilepsy.

In addition, it is appreciated that the structural homology between the neuronal Na and Ca channels extends to their counterparts in other cells, among them cardiac cells, vascular and skeletal smooth muscle cells, pituitary cells, and secretory cells within the pancreas, pituitary, and adrenal medulla (see above and Hess, P., *Ann. Rev. Neurosci.* **13**, 337-356). For example, as demonstrated in **Example 7**, a subset of compounds of the invention effectively block L-type Ca channels of clonal pituitary

secretory cells; said L-type Ca channels are in turn closely related to cardiovascular L-type channels. Accordingly, compounds of the invention will be useful in therapy of a variety of additional disorders treatable by block of said Ca and Na channels. Examples of said disorders are further discussed herein, among them: cardiovascular disorders such as cardiac arrhythmias and hypertension; and disorders of secretion such as acromegaly or diabetes insipidus. Applying the same rationale, compounds of the invention will be useful for other indications, among them relief of chronic pain and as local anesthetics.

Voltage-activated Na-channels to be blocked by said compounds include but are not limited to voltage-activated ('action potential'), tetrodotoxin-sensitive Type I and Type II sodium channels in nerve axons and nerve terminals, and voltage-activated tetrodotoxin-insensitive sodium channels in cardiac or skeletal muscle [Catterall, W.A. (1992) Physiol. Rev. **72**, S15-S48]. Voltage-activated Ca-channels to be blocked by said compounds include but are not limited to voltage-activated L-type calcium channels of heart, skeletal muscle, or brain; voltage-activated N-type calcium channels of neurons or neuroglial cells; and voltage-activated P or- Q-type calcium channels of nerve terminals, or nerve cell bodies [Snutch, T.P. and Reiner, P.B. (1992) Curr. Opin. Neurobiol. **2**, 247-53; Tsien, R.W. et al (1991) Trends Pharmacol. Sci. **12**, 349-54; Zhang, J.-F. et al. (1993) Neuropharmacol. **32**, 1075-84].

B. Uses of compounds of the invention as neuroprotective agents.

Because of the structural homologies of the SS1/SS2 ion-selectivity filter regions among the major subclasses of voltage-activated Ca and Na channels, it has been possible for us to create compounds of the invention with a broad spectrum of action on the major subclasses of neuronal voltage-activated Na and Ca channels, as demonstrated herein. This broad spectrum of action is desirable for treatment a variety of disorders which involve excessive activation of said channels, among them brain ischemia and epilepsy. We demonstrated herein the creation of effective blockers of glutamate

release, with substantial neuroprotective activity *in vivo*, which possess dual actions at voltage-activated Ca and Na channels: blockade of presynaptic Ca channels and blockade of neuronal TTX-sensitive Na channels. Thus we have achieved, as a result of our novel strategies for drug discovery, the creation of a compound series that combines some of the most desirable properties of several of the most promising classes of neuroprotective antagonists of voltage-activated ion channels: a class of substituted guanidines that are use-dependent blockers acting at a site common to both presynaptic Ca channels and neuronal Na channels.

Compounds of the invention have been described with dual actions at neuronal voltage-activated Ca and Na channels; among them CNS 1237, are effective use-dependent blockers of voltage-activated ion channel activity and concomitant neurotransmitter release from brain nerve terminals induced by sustained depolarization **(Examples 7, 4 and 8)** as well as by said activity induced by brief depolarizations over a wide range of frequencies **(Examples 2 and 3)**. The use-dependent block of voltage-activated Na and/or Ca channels exhibited by compounds of the invention will be useful under the aforementioned pathophysiological circumstances of brain- and tissue-ischemia. While not wishing to be bound by theory, the following is a reasonable explanation for the therapeutic efficacy of compounds of the invention:

Under the circumstances of brain or tissue ischemia, which result in excessive and/or inappropriate depolarization of the excitable cell membrane, compounds of the invention will exhibit effective blockade of said ion channels. This results in reduced Ca and/or Na influx into the cells through said ion channels within the cell membrane, hence avoiding the concomitant pathophysiological consequences. Among the avoidable consequences are cell injury and death induced by excessive Ca entry as occurs in brain ischemia, and inappropriately timed activation of contractions of the heart by Na channel activation as occurs in cardiac arrhythmias generated in ischemic cardiac tissue.

In contrast, compounds of the invention are relatively ineffective in blocking said ion channels in healthy tissue, because the cell membrane in which said ion channels reside is more usually hyperpolarized, causing the ion channels to adapt conformational states which are resistant to interaction with said compounds of the invention.

Efficacy as therapy in stroke is exemplified by the actions of CNS 1237, which effectively reduces cell death in a rodent models of focal ischemia (stroke) (**Figure 12** and **Example 9**). As shown in **Example 10 and Figure 13**, compounds of the invention with such dual actions (CNS 1237, CNS 5149, and CNS 1217) are neuroprotective in an animal model relating to depolarization-induced neuronal hyperactivity such as occurs in epilepsy.

Said compounds may be administered prophylactically or within 72 hours of an acute ischemic insult to prevent cellular death and destruction. We anticipate that for limitation of cellular damage and destruction triggered by an ischemic insult, treatment should preferably commence within 24 hours of said insult.

C. Uses of compounds of the invention for therapy of disorders of secretion

Secretion of a variety of substances from substances from non-neuronal secretory cells occurs by a process of Ca-dependent exocytosis closely resembling the mechanism of Ca-dependent neurotransmitter release [Rubin, R.P. (1970) Pharmacol. Rev. **22**: 389-428]. Examples of this include release of norepinephrine from adrenal chrommaffin cells [Landsberg, L. and Young, J.B. (1987) in Harrison's Principles of Internal Medicine, 11th Ed., Braunwald et al. eds., New York, McGraw-Hill, pp. 358-370; Neher, E. and Zucker, R.S. (1993) Neuron **10**, 21-30], secretion of peptide hormones from the pituitary [Tse et al. (1993) Science 260, 82-84; Chang, J.P. et al.

(1988) Endocrinology **123**, 87-94], secretion of digestive enzymes from pancreatic acinar cells [Muallem, S. (1989) Ann. Rev. Physiol. **51**: 83-105], and secretion of insulin from pancreatic beta cells [Larner, J. (1985) in *The Pharmacological Basis of Therapeutics, 7th Ed.*, Goodman, L.S. and Gilman, A.G. eds., New York, MacMillan, pp. 1490-1503]. The voltage-activated Ca channels that play a major role in governing said processes resemble those that govern neurotransmitter release, in terms of structure, pharmacology, and mechanism [Bean, B.P. (1989) *Ann. Rev. Physiol.* **51**: 367-384; Hess, P. (1990) Ann. Rev. Neurosci. **13**:337-56; Cohen, C.J., and McCarthy, R.T. (1987) *J. Physiol.* **387**: 195-225]. Accordingly the ability of compounds of the invention to block neurotransmitter release as described in Example 29, and the ability of said compounds to block the activity of voltage activated Ca channels described in **Examples 6 and 7**, constitutes strong evidence that compounds of the invention will be effective inhibitors of exocytotic secretion of a variety of substances from a variety of non-neuronal cells. In particular, **Example 7** documents the ability of compounds of the invention to block Ca channels in GH4C1 clonal pituitary cells. Said pituitary cells secrete prolactin and growth hormone [Tashjian, A.H. (1979) in Meth. Enyzmol. 58: 527-535, Acad. Press, N.Y.]. Block of voltage-activated Ca channels of pituitary cells GH4C1 cells inhibits secretion of said peptides [Tan, K.N., and Tashjian, A.H. (1984) J. Biol. Chem. **259**: 418-426]. Therefore, the results disclosed in **Example 7** constitute a direct demonstration of the ability of compounds of the invention to function as effective antisecretory agents.

The aforementioned ability of compounds of the invention to block Ca channels which are identical or closely related to those which regulate secretion, indicate that compounds of the invention will find utility in therapy of secretory disorders. An example of a hypersecretory disorder treatable by a suitably formulated compound of the invention could be the treatment of pheochromocytoma, which is a disorder resulting from the presence of a tumor of the chromaffin cells in the adrenal medulla [Bravo, E.L. and Gifford, R.W. (1984) *New Eng. J. Med.* **311**: 1298-1300]. This

disorder is characterized by the hypersecretion of catacholamines, resulting in hypertension which may be paroxysmal and associated with attacks of palpitation, headache, nausea, breathing difficulty, and anxiety. Another hypersecretory disorder treatable by compounds of the invention may be pancreatitis, which is an inflammation of the pancreas leading to hypersecretion of hormones and enzymes from the acinar cells of the pancreas, among them hormones such as vasoactive intestinal peptide (VIP) and insulin; digestive enzymes and their inactive precursors, among them lipases and proteases, deoxyribonucleases, ribonucleases, and amylase [Greenberger, N.J. et al.(1987) in *Harrison's Principles of Internal Medicine, 11th Ed.*, New York, McGraw-Hill, pp. 1372-1380] . In severe cases of pancreatitis, autodigestion of the pancreas by the hypersecretion and subsequent activation of said digestive enzymes can be fatal Greenberger, N.J. et al., *ibid*]. For these and other indications mediated by hypersecretory activity outside the central nervous system, compounds of the invention, particularly those which are charged and/or hydrophilic and otherwise do not cross the blood/brain barrier are believed to be clinically useful upon systemic and/or local administration.

Certain hypersecretory disorders may result from abnormal activity of cells within the central nervous system, among them cells of the pituitary gland, also termed the hypophysis, located at the base of the brain. Secretion of hormones and related substances from cells of the adenohypohysis is regulated by releasing factors, primarily those secreted by the hypothalamus [Cooper, P.E. and Martin, J.B. (1992) in *Diseases of the Nervous System: Clinical Neurobiology*, Asbury et al. eds, Saunders, Philadelphia, pp. 567-583]. Substances secreted by the adenohypophysis include growth hormone, prolactin, thyroid stimulating hormone (TSH), and adrenocorticotrophic hormone (ACTH). Hypersecretion of these substances from the pituitary can lead to a variety of disorders of growth (e.g. acromegaly due to hypersecretion of growth hormone) and metabolism (e.g. secondary hyperthyroidism triggered by hypersecretion of TSH, and Cushing's disease, which results from

excessive secretion by the pituitary of precursor peptides containing ACTH) [see Cooper, P.E., and Martin, J.B. *ibid*.]. These disorders are often due to benign tumors of the pituitary secretory cells. It is our belief that appropriate pharmacotherapy with suitable compounds of the invention, preferably those which are hydrophobic and/or by some means penetrate the blood brain barrier, may be of value for such disorders. In some instances pharmacotherapy with compounds of the invention may obviate the necessity and attendant risk of neurosurgery performed for the purpose of removing such benign tumors.

Secretion from the neurohypophysis of the pituitary is regulated by innervation from elsewhere in the CNS. For example, the release of oxytocin and vasopressin is regulated at least in part by the activity of neurons in the paraventricular nucleus of the hypothalamus, which innervates the neurohypophysis. Compounds of the invention will be useful in disorders of secretion of substances from the neurohypophysis, among them dilutional hyponatremia, which is believed to be caused by inappropriate secretion of vasopressin [see Martin, J.B., and Reichlin, S., *Clinical Neuroendocrinology*, 2nd Edition (1987) Philadelphia, Davis].

By the aforementioned rationale, compounds of the invention will also be useful for treatment of disorders involving hypersecretion of substances produced by the hypothalamus, among them diabetes insipidus, which may be caused by hypersensitivity to, or excessive release of, AVP. AVP is a peptide synthesized in and released from neurons of the supraoptic and paraventricular nuclei of the hypothalamus [see Cooper, P.E., and Martin, J.B. in *Diseases of the Nervous System: Clinical Neurobiology*, Asbury et al., eds, Saunders, Philadelphia (1992), pp. 567-583]. A current means of treatment of diabetes insipidus is surgical destruction of most of the cells in the supraoptic nucleus. Pharmacotherapy with compounds of the invention may in some instances obviate the need for such neurosurgery.

D. Use of compounds of the invention for treatment of cardiovascular disorders

The aforementioned ability of compounds of the invention to block Ca channels which are identical or closely related to those which regulate cardiovascular function, as documented in **Example 7**, indicate that compounds of the invention will find utility in therapy of cardiovascular disorders. Among the disorders currently known to be treatable by inhibitors of L-type Ca channels such as verapamil, diltiazem, and nifedipine are hypertension, angina pectoris, cardiac arrhythmias. As shown in **Example 7**, a subset of compounds of the invention show equal or greater potency for block of L-type Ca channels when compared with the ability of verapamil or diltiazem to block said channels using the same assay protocol.

The aforementioned ability of compounds of the invention to block Na channels which are closely related to those which regulate cardiovascular function, as documented in **Example 8**, indicate that compounds of the invention will find utility in therapy of cardiovascular disorders treatable by blockers of Na channels. A major indication for such Na channel blockers is cardiac arrhythmias, which are currently treated by blockers of Na channels; among them quinidine, procainamide, lidocaine, and diphenylhydantoin (phenytoin). Among the cardiac arrhythmias successfully treatable by said Na channel blockers are ventricular tachycardia; ventricular premature depolarizations; digitalis-induced atrial tachycardia and atrial and ventricular arrhythmias; paroxysmal supraventricular tachycardia; atrial fibrillation; and prophylaxsis against the development of supraventricular arrhythmias [see Bigger, J.T., and Hoffman, B.F. (1985) in *The Pharmacological Basis of Therapeutics, 7th Ed.*, Goodman, L.S. and Gilman, A.G. eds., New York, MacMillan, pp. 748-783]. Accordingly, compounds of the invention should find utility in treatment of cardiac arrhythmias treatable by blockers of cardiac Na channels. For these and other indications treatable by blocking cardiac Na channel activity, compounds of the invention, particularly those which are charged and/or hydrophilic and otherwise do not

cross the blood/brain barrier, are believed to be clinically useful upon systemic and/or local administration.

In light of the fact that some cardiac arrhythmias, among them paroxysmal supraventricular tachycardia and other supraventricular arrhythmias, are treatable by both blockers of cardiovascular L-type channels and by blockers of cardiac Na channels, we believe that compounds of the invention with dual actions against Na channels and Ca channels may have particular utility in treatment of said arrhythmias. Compounds of the invention possessing such dual actions, among them CNS 1217 and CNS 5127, are illustrated by the results described in **Example 7** and **Example 8**.

E. Mode of administration of compounds of the invention

Compounds of the invention can be employed, either alone or in combination with one or more other therapeutic agents as discussed above, as a pharmaceutical composition in mixture with conventional excipient, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral or intranasal application which do not deleteriously react with the active compounds and are not deleterious to the recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the active compounds.

For parenteral application, particularly suitable are solutions, preferably oily or aqueous solutions as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or carbohydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

Intravenous or parenteral administration, e.g., sub-cutaneous, intraperitoneal or intramuscular administration are generally preferred. It will be appreciated that the actual preferred amounts of active compounds used in a given therapy will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, the particular site of administration, etc. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines. In general, a suitable effective dose of one or more compounds of the invention, particularly when using the more potent compound(s) of the invention, will be in the range of from 0.5 to 500 milligrams per kilogram bodyweight of recipient per day, preferable in the range of 1 to 100 milligrams per kilogram bodyweight of recipient per day. The desired dose is suitable administered once daily, or in several sub-doses, e.g., 2 to 4 sub-doses, are administered at appropriate intervals through the day, or other appropriate schedule. Such sub-doses may be administered as unit dosage forms, e.g., containing from 0.25 to 25 milligrams of compound(s) of the invention per unit dosage, preferably from 0.5 to 5 milligrams per unit dosage. Alternatively, compounds of the invention may be administered continuously for a period of time, for example by an intravenous infusion or by means

of a transdermal mode of delivery using, for example, a patch incorporating and releasing said compounds.

It is appreciated that, in view of the structural and pharmacological similarities among the subclasses of Na channels and the subclasses of calcium channels that comprise the preferred molecular targets of compounds of the invention, instances may arise in which site-specific drug-delivery methods would constitute a preferred method of delivering compounds of the invention to the tissue in need of therapy [See Tomlinson, E., (1987) Advanced Drug Delivery Reviews **1**, 87-198]. For example, in the case of disorders of muscle function originating from a pathophysiological condition of Na channels of skeletal muscle, among them hyperkalemic periodic paralysis, it may be desirable to microencapsulate compounds of the invention within delivery vehicles such as liposomes [Yagi, K. (1986) *Medical Applications of Liposomes*, Japan Soc. Press, Tokyo; Gregoriadis, G., ed. (1984) Liposome Technology, Vol. I-III, CRC press, Inc., Cleveland], said liposomes containing a monoclonal antibody targetted to specific antigens on or near the surface of the diseased muscle cells. Said method of drug delivery should result in selective binding of the liposomes to the target tissue, and release of the compound of the invention near the abnormally functioning skeletal muscle Na channels, where said compound will inhibit the persistent activation of muscle Na channels which constitutes the molecular abnormality underlying the disease. The use of site-directed pro-drugs or related chemical modifications of compounds of the invention may enable a lower dosage, and/or result in fewer undesirable side effects.

Another instance in which such a targetted drug delivery method may be desirable is in the case of pheochromocytoma or another abnormality which results in hypersecretion of catecholamines into the blood. Because the Ca channels of chromaffin cells are closely related to those of nerve, cardiac cells, and muscle (Neher, E. and Zucker, R.S. (1993) Neuron **10**, 21-30; Bean, B.P. (1989) *Ann. Rev. Physiol.* **51**:

Example 1

Amino acid sequences of the SS1/SS2 ion-selectivity filter domains for a family of Na and Ca channel targets comprising sites of direct interaction with compounds of the invention.

	SOURCE	SS1/SS2 REGION SEQUENCE	RES. #'s
Repeat I			
Na channel	Human cardiac[1]	R L M T Q D C W E R L Y Q	367-379
	Human skeletal[2]	R L M T Q D Y W E N L F Q	401-414
	Brain II[8]	R L M T Q D F W E N L Y Q	379-391
	Brain III[9]	R L M T Q D Y W E N L Y Q	378-390
	Heart I[10]	R L M T Q D C W E R L Y Q	368-380
	Skeletal μ1[11]	R L M T Q D Y W E N L F Q	395-407
Ca channel	Human a1D[3]	Q C I T M E G W T D V L Y	359-371
	Rat brain,rbA[4]	Q C I T M E G W T D L L Y	315-327
	Rat brain,rbB[5]	Q C I T M E G W T D I L Y	309-321
	Rat brain,rbCI[6]	Q C I T M E G W T D V L Y	358-370
	Rat brain[7]	Q C I T M E G W T D V L Y	358-370
	Brain BI[13]	Q C I T M E G W T D L L Y	313-325
	Cardiac[14]	Q C I T M E G W T D V L Y	388-400
	Skeletal[15]	Q C I T M E G W T D V L Y	287-299
Repeat II			
Na channel	Human c[1]	R I L C G E W I E T M W D	893-905
	Human s[2]	R I L C G E W I E T M W D	756-768
	Brain II[8]	R V L C G E W I E T M W D	937-949
	Brain III[9]	R V L C G E W I E T M W D	889-901
	Heart I[10]	R I L C G E W I E T M W D	896-908
	Skeletal μ1[11]	R I L C G E W I E T M W D	750-762
Ca channel	Human a1D[3]	Q I L T G E D W N A V M Y	700-712
	Rat brain,rbA[4]	Q I L T G E D W N E V M Y	665-677
	Rat brain,rbB[5]	Q I L T G E D W N A V M Y	658-670
	Rat brain,rbCI[6]	Q I L T G E D W N S V M Y	701-713
	Rat brain[7]	Q I L T G E D W N A V M Y	699-711
	Brain BI[13]	Q I L T G E D W N E V M Y	663-675
	Cardiac[14]	Q I L T G E D W N S V M Y	731-743
	Skeletal[15]	Q V L T G E D W N S V M Y	609-621

367-384; Hess, P. (1990) Ann. Rev. Neurosci. 13:337-56], systemic administration of a compound of the invention at concentrations sufficient to block release of catecholamines from chromaffin cells may produce undesirable side effects resulting from block of, for example, neuronal and cardiovascular Ca channels. Accordingly, delivery of compounds of the invention to chromaffin cells may be enhanced by their incorporation into liposomes containing a monoclonal antibody targetted to specific antigens on or near the surface of the chromaffin cells of the hypersecreting adrenal medulla.

This method of liposome-mediated drug targetting is currently being developed for delivery of a variety of agents, to be used for indications such as cancer chemotherapy and destruction of tumors [e.g., Bassett, J.B. et al. (1986) J. Urol. 135, 612-615]. It is recognized that refinements of the above method and many new methods for site-specific drug delivery are constantly being developed, and we do not in any way intend to be bound by the current state of the art of drug delivery. Appropriate methods for site-specific delivery of compounds of the invention may include incorporation of said compounds into polymer beads which afford slow site-specific release [Mathiowitz, E., and Langer, R. (1987), J. Controlled Release 5, 13-18], and delivery to the target tissue by means of surgically implanted pumps.

The following examples illustrate the invention and are not intended to be limiting in any way.

	SOURCE	SS1/SS2 REGION SEQUENCE	RES. #'s
Repeat III			
Na channel	Human c[1]	Q V A T F K G W M D I M Y	1414-1426
	Human s[2]	Q V A T F K G W M D I M Y	1239-1251
	Brain II[8]	Q V A T F K G W M D I M Y	1417-1429
	Brain III[9]	Q V A T F K G W M D I M Y	1363-1375
	Heart I[10]	Q V A T F K G W M D I M Y	1416-1428
	Skeletal μ1[11]	Q V A T F K G W M D I M Y	1232-1244
Ca channel	Human a1D[3]	T V S T F E G W P A L L Y	1096-1108
	Rat brain,rbA[4]	T V S T G E G W P Q V L K	1406-1418
	Rat brain,rbB[5]	T V S T G E G W P M V L K	1362-1374
	Rat brain,rbCI[6]	T V S T F E G W P E L L Y	1110-1122
	Rat brain[7]	T V S T F E G W P A L L Y	1095-1107
	Brain I[13]	T V S T G E G W P Q V L K	1464-1476
	Cardiac[14]	T V S T F E G W P E L L Y	1140-1152
	Skeletal[15]	T V S T F E G W P Q L L Y	1009-1021
Repeat IV			
Na channel	Human c[1]	Q I T T S A G W D G L L S	1706-1718
	Human s[2]	E I T T S A G W D G L L N	1531-1543
	Brain II[8]	Q I T T S A G W D G L L A	1709-1721
	Brain III[9]	Q I T T S A G W D G L L A	1655-1677
	Heart I[10]	Q I T T S A G W D G L L S	1708-1720
	Skeletal μ1[11]	E I T T S A G W D G L L N	1524-1536
Ca channel	Human a1D[3]	R C A T G E A W Q E I M L	1401-1413
	Rat brain,rbA[4]	R S A T G E Q W H N I M L	1702-1714
	Rat brain,rbB[5]	R S A T G E A W H E I M L	1650-1662
	Rat brain,rbCI[6]	R C A T G E A W Q D I M L	1411-1423
	Rat brain[7]	R C A T G E A W Q E I M L	1400-1412
	Brain BI[13]	R S A T G E A W H N I M L	1760-1772
	Cardiac[14]	R C A T G E A W Q D I M L	1441-1453
	Skeletal[15]	R C A T G E A W Q E I L L	1318-1330

Alignment of the amino-acid sequences (in single-lettercod) in the regions encompassing the SS2 segment of the four repeats of different human and rat sodium and calcium channels. The cluster that contains the residues identified as determinants of Ion selectivity is highlighted, and serves as a point of alignment for the homologous channel sequences within each of the repeats. The superscripts above the source identify the type of channel and the reference from which the sequence was obtained. The numbers of the amino-acid residues in each sequence are given on the right-hand side.

Alignment comments/references:

(1) human cardiac TTX-insensitive, voltage-dependent sodium channel
Gellens, M. E., A. L. George, L. Chen, M. Chahine, R. Horn, R. L. Barchi and R. G. Kallen (1
Proc. Natl. Acad. Sci. USA 89, 554-558.

(2) human skeletal muscle sodium channel
George, A. L., J. Komisarof, R. G. Kallen and R. L. Barchi (1992) Ann. Neurol.31, 131-137.

(3) human neuronal (neuroblastoma) calcium channel; a1D subunit
Williams, M. E., D. H. Feldman, A. F. McCue, R. Brenner, G. Belicelebi, S. B. Ellis and M. M
Harpold (1992) Neuron 8, 71-84.

(4) rat brain calcium channel; a1 subunit, rbA form
Starr, T. V. B., W. Prystay and T. P. Snutch (1991) Proc. Natl. Acad. Sci. USA 88, 5621-562

(5) rat brain ω-CTX sensitive calcium channel; a1 subunit, rbB-I form
Dubel, S. J., T. V. B. Starr, J. Hell, M. K. Ahlijanian, J. J. Enyeart, W. A. Catterall and T. P. S
(1992) Proc. Natl. Acad. Sci. USA 89, 5058-5062.

(6) rat brain voltage-dependent calcium channel;a1 subunit, rbC-I form (the rbC-II isoform has identical sequence to rbC-I in these regions)
Snutch, T., W. J. Tomlinson, J. P. Leonard and M. W. Gilbert (1991) Neuron 7, 45-47.

(7) rat brain voltage dependent calcium channel; a1 subunit
Hui, A., P. T. Ellinor, O. Krizanova, J-J. Wang, R. J. Diebold and A. Schwartz (1991)
Neuron 7, 35-44.

(8) rat brain sodium channel II
Noda, M. et al. (1986) Nature 320, 188-192.

(9) rat brain sodium channel III
Kayano, T., M. Noda, V. Flockerzi, H. Takahashi and S. Numa (1988) FEBS Lett. 228,
187-194.

(10) rat heart sodium channel I
Rogart, R. B., L. L. Cribbs, L. K. Muglia, D. D. Kephart and M. W. Kaiser (1989) Proc. Natl.
Acad. Sci. USA 86, 8170-8174.

(11) skeletal muscle μI sodium channel
Trimmer, J. S. et al. (1989) Neuron 3, 33-49.

(12) electrophorous electroplax sodium channel (sequence not shown)
Noda, M. et al. (1984) Nature 312, 121-127.

(13) rabbit brain Ca channel BI
Mori, Y. et al. (1991) Nature 350, 398-402.

(14) rabbit cardiac L-type Ca channel
Mikami, A. et al. (1989) Nature 340, 230-233.

(15) rabbit skeletal muscle L-type Ca channel
Tanabe, T. et al. (1987) Nature 328, 313-318.

Example 2

Use-dependent block by compounds of the invention for voltage-activated ion channels.

Summary of Experimental Strategy: Resolution of Tonic and Frequency-dependent blockade of sodium current through type II neuronal voltage-activated sodium channels:

Antagonists of neuron-specific type II subclass of voltage-gated Na channels are neuroprotective [Sys P.K., S.G. Waxman, and B.R. Ransom (1992) *J. Neurosci.* **12**, 430-439]. The ability of compounds of the invention to block voltage-activated type II Na channels was determined in a functional assay employing Chinese hamster ovary cell line expressing cloned type II Na channels (CNaIIA-1) derived from rat brain [West, J.W., T. Scheuer, L. Maechler, and W.A. Catterall (1992) *Neuron* **8**, 59-70]. This assay is based on the observation that the Na channel exists in three functional states: resting, open, and inactivated (ref.?). Membrane depolarization opens resting Na channels, allowing an inward flow of Na current. Open Na channels then quickly inactivate and return to the resting state. Various Na channel antagonists can interact with one or more of these functional states and block Na current.

Accordingly, the assay utilizes the whole cell configuration of the voltage clamp recording technique (Hamill *et al.*, 1981) which allows a direct measurement of inward sodium current. The assay takes advantage of the fact that varying the timing of stimuli relative to the application Na channel antagonists provides a methodology for distinguishing between tonic (resting state) and frequency-dependent (open state) blockade of sodium channels. The protocol of the assay is described below.

Assay protocol:

The CNaIIA-1 cells are grown in RPMI 1640 (MediaTech) supplemented with 5% FCS (Hyclone), 200 ug/ml G418 (Sigma) and 5.75 mg/ml Proline (Sigma). EDTA-free trypsin (1:250, Sigma) is used to split the cells 1X per week and cells are seeded @ 1:100 into a T75 flask (CoStar) and 1:200 - 1:6400 into a 24-well plate containing glass coverslips (Fisher). The media is changed once a week in the flasks. Since a single 24-well plate is prepared each week, the media is not changed. Cells whose passage number exceeds 20-25 are not used.

The external recording solution contains 150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Glucose, 5 mM HEPES; pH 7.4, adjusted with NaOH; ~310 mOsm. Working solutions are prepared from 10X stock solutions. The internal pipette solution contains 150 mM CsF, 10 mM EGTA, 10 mM HEPES; pH 7.4, adjusted with CsOH; ~300 mOsm.

Whole cell currents are measured using an Axopatch 200A integrating patch clamp amplifier (Axon Instruments; Foster City, CA. Capacity transients are cancelled and series resistance is compensated 90%. Inward sodium currents are elicited by stepping from a holding potential of -90 mV to a test potential of 0 mV. Isolated cells are used whenever possible to eliminate artifacts caused by gap junctions and/or poor spatial clamping.

An experimental protocol was developed to differentiate between a compound's ability to induce tonic block (blockade of the resting state) and its ability to produce frequency-dependent block (block of the open state) of sodium channels. Tonic versus frequency-dependent block was determined by first recording a series of control whole-cell sodium currents evoked by the stimulus protocol described above delivered at a rate of 1 Hz (Fig.xA). The drugs were then bath applied to the recording chamber (volume = 0.75 cc) at a rate of 2 ml/min for a period of 2 min in the absence of stimulation . A second series of test pulses was delivered (again at 1 Hz) in the presence of the drug.

The drugs were then washed out and the cell allowed to fully recover. The entire regime was then repeated at 10 Hz. Relative current from each trial (1 trial = 10 pulses @ 1 Hz or 40 pulses @ 10 Hz) was then normalized by either dividing the current amplitude recorded from the first pulse of the test train (I_{first}) by the average current recording during the control train ($I_{ave.\ control}$) for determination of tonic block (Eq. 1) or by dividing all the current amplitudes of a given trial (I_{pulse}) by the highest amplitude of that trial ($I_{max\ pulse}$) for frequency-dependent block (Eq. 2).

Eq. 1. $\text{Relative current}_{tonic} = (I_{first}/I_{ave.\ control})$

Eq. 2. $\text{Relative current}_{frequency\text{-}dependent} = (I_{pulse}/I_{max\ pulse})$

Relative current_{tonic} and relative $\text{current}_{frequency\text{-}dependent}$ were then plotted together as a function of time and the resulting graph was fitted by the single exponential function shown in Eq. 3 (Fig. xB).

Eq. 3. $y = a * exp\ (-x/t) + b,$

where t is the time constant of the onset of block and b is the maximum degree of block.

Resolution of depolarization-dependent enhancement of the potency and/or efficacy of block of voltage-activated Na channels

Figure 9 demonstrates the ability, enhanced by depolarization, of one of the compounds of the invention, CNS 1237, to block voltage-sensitive type II neuronal Na channels. As the repetitive-depolarization-("frequency")-dependent block curves are to the left of the tonic block curve, CNS 1237 is considered to preferentially block the open state of the Na channel over the resting state. Therefore, it is concluded that CNS

1237 is better at producing frequency-dependent block than it is at producing tonic block. This characteristic is contrasted with the inability of one of the compounds of the prior art, tetrodotoxin (TTX), to produce the same phenomenon when tested in an identical manner. The tonic block curve for TTX is shifted to the left of the frequency-dependent curves, which overlap each other. Therefore it is concluded that TTX is better at producing tonic block of Na channels than it is at producing frequency-dependent block. The above tonic/frequency-dependent block relationship generates a fingerprint identifying compounds on the basis of their relative preference for producing one type of Na channel blockade over another.

In some cases, certain compounds required tens of minutes for their effects on sodium current to reverse. Those compounds proved not to very amenable to the tonic/frequency-dependent block protocol described above. In cases where slowly-reversible compounds were encountered, the protocol was modified in the following way: stimulus rates of 0.1 and 1 Hz were used and cells were superfused with drug in the absence of stimulation for a period of at least three minutes. The results from a slowly-reversible compound are shown in Figure 13.

Example 3

Use-dependent enhancement f the rate of ion channel bl ck by comp unds of the invention.

Summary of Experimental Strategy: Resolution of Frequency-dependent acceleration of the onset of blockade of sodium current through type II neuronal voltage-activated sodium channels:

Depolarization-dependent block of voltage-activated sodium current in a single voltage-clamped CNaIIA-1 cell. Currents were elicited by 10 ms depolarizations to 0 mV from a holding potential of -90 mV. Current amplitudes have been normalized such that the value obtained in a standard saline solution at the start of the experiment is 1.0. After measuring the control currents, CNS 5149 (10 M) was bath-applied for 2 minutes in the absence of stimulation. Stimulation was then resumed at 0.03 Hz in the continued presence of the compound (n.). The current in response to the first such stimulus (at time zero on the graph) is reduced compared to control currents, reflecting tonic (depolarization-independent) blockade (Eq. 1). Additional slowly accumulating block (fitted by Eq. 2) develops with further stimulation. This cell was washed extensively with compound-free saline, and the experiment repeated using a stimulus frequency of 0.1 Hz (l). The rate of block was faster at the higher stimulation frequency (Eq. 3). Since steady-state blockade takes 3 to 5 minutes to develop, drug action is considered to be slow.

The behavior of representative compounds of the invention is illustrated in **Figure 13,** which provides examples of each of slow and fast depolarization-dependent blockade of sodium current.

Panel **A, Figure 13.** Blockade induced by CNS 5149. Methods as in Example 4, except that three stimulation frequencies were used: 0.03 Hz (n.), 0.1 Hz (l), and 10 Hz (t) The effect of the compound develop slowly even when the rate of applied depolarizations is as high as 10 Hz .

Panel **B, Figure 13.** In contrast, steady-state block with CNS 1217 is reached within a few seconds. Methods as in Example 4, except that stimulation frequencies of 1.0 Hz (n.) and 10 Hz (s) were used. The onset of block is again more rapid at the faster stimulation rate.

Panel **C, Fig.13.** Tonic and depolarization-dependent block of sodium current by 100 M TTX in a single CNaIIA-1 cell. Methods as in Example 4. Tonic blockade is 60% of control sodium current. There is only a very small additional degree of blockade mediated by depolarization- dependent mechanisms. The reduction in current is only 15% and there is no difference in rate of onset or degree of steady-state block as a function of stimulus rate (1 Hz vs. 10 Hz). Data fitted by the function in Equation 3 of the previous example.

Example 4

Use-dependent acceleration of the decay of Ca channel-mediated glutamate release from brain nerve terminal preparations

Details of experimental strategy

We have adapted a novel rapid superfusion system [S. Goldin (1990) U.S. Patent #4,891,185; Turner, T.J. Bruce, L.B., and Goldin (1989) Anal. Biochem. **178**:8-16] to measure depolarization-induced ^{3}H-glutamate release from brain nerve terminals. The depolarizing stimulus opens presynaptic voltage-activated ion channels as the key step required to initiate Ca-dependent exocytosis of glutamatergic synaptic vesicles. The method, involves preloading rat brain synaptosomes with ^{3}H-glutamate via the Na-dependent glutamate uptake system. The preloaded nerve terminals are retained in a superfusion chamber accessed by high-speed, solenoid-driven valves. Microcomputer-operated circuitry controls the timing of valve operation; the valves control the delivery under nitrogen pressure of pulses of depolarizing buffer, Ca, and/or drugs to the synaptosomes. The ^{3}H-glutamate-containing effluent is continuously collected in a high speed fraction collector on a subsecond timescale as short as 30 msec (300 msec fractions were employed herein, and in **Figures 8 and 11**). The high solution flow rate and minimal dead volume of the superfusion chamber, afford rapid solution changes and precise control of the chemical microenvironment of the nerve terminal preparation.

The method is, more specifically, that described in Goldin et al., PCT/US92/01050, with some minor modifications. In said method, introduction of a buffer containing high [K^+] was the means employed to produce the depolarization. This mode of depolarization remains the preferred method of opening presynaptic voltage-activated Ca channels to trigger glutamate release. However, in the current example an additional method of depolarization, namely introduction of veratridine,

was employed in experiments performed in parallel. Veratridine is known to stimulate neurotransmitter release by opening voltage-activated Na channels, which results in depolarization of the nerve terminal plasma membrane and in turn, secondarily, opens presynaptic Ca channels to directly trigger ^{3}H-glutamate release via Ca-dependent exocytosis [Leach et al., Epilepsia **27**, 490-497]. The use of veratridine-induced glutamate release was employed to detect compounds of the invention which may block neurotransmitter release by blocking voltage-activated presynaptic Na channels. It has been previously demonstrated that tetrodotoxin, a highly specific blocker of Type I and Type II neuronal Na channels, blocks veratridine-induced ^{3}H-glutamate release with no effect on high $[K^+]^-$ induced ^{3}H-glutamate release [Katragadda et al. (1993) Abs. Soc. for Neurosci. **19**, 1750]. In experiments measuring veratridine-induced glutamate release, 50 µM veratridine in "basal" buffer was substituted for the "high-K buffer" employed as described in PCT/US92/01050; the protocol was otherwise identical to that described therein. In superfusion solutions containing compounds to be tested, compounds were made as stock solutions in methanol and diluted so that the final concentration of methanol never exceeded 0.3% (v/v). All solutions including compound free controls contained the same [methanol].

Use-dependent acceleration of the decay of Ca channel-mediated glutamate release

The ability of compounds of the invention block glutamate release from brain nerve terminal preparations is illustrated in **Figure 8**. As shown in **Figure 8a,** a compound of the invention, CNS 1237, accelerates the decay and reduces the amplitude of Ca-dependent ^{3}H-glutamate release. Rat brain nerve terminals, loaded with ^{3}H-glutamate, were superfused with High-K^+ buffer (55 mM K^+) with or without Ca+2 (2.4 mM). Similar experiments were also done with 1 M and 2 M CNS 1237 in superfusion buffers. Experiments with out Ca^{+2} were subtracted from the corresponding experiments with Ca^{+2} to obtain Ca-dependent release. Release was

expressed as % of specific 3H-glutamate uptake by nerve terminals. Ca-dependent release was fitted to a single exponential decay to ($Y=A*\exp(-X/\tau)$ using the modeling software 'RS/1'. In this experiment superfusion was done at a much longer time scale compared to the earlier experiments to be able to follow the entire decay of persistent component thus the rapidly decaying phasic component is not resolved. Fractions were collected at 0.3 sec intervals. These longer fractions mask the phasic release.

As demonstrated in **Figure 8b**, substituted guanidine compounds of the invention show use dependence in blocking Ca-dependent glutamate release. Initial amplitude and decay constants of Ca-dependent release in the presence of drug were expressed as % of control (no CNS compound in superfusion). The results are from a minimum of 3 different experiments of each condition. CNS compounds were tested at 2 M with the exception of 5149 (1 M) and 5118 (3 M). ω-Aga IV A and w-Cm toxin MVIIC were tested at 0.3 M.

Example 5

Block by compounds of the invention of the SS1/SS2 ion selectivity filter of voltage-activated ion channels.

Solid evidence has been obtained, using the radiolabled TTX analogue ^{3}H saxitoxin (93) that CNS 1145 and CNS 1237 interact directly with the ion-selectivity filter region of Na channels. [^{3}H]STX binding was measured in rat brain synaptosomes essentially as previously described (130). Synaptosomal protein was incubated with 6 nM [^{3}H]STX, in the presence or absence of tested compounds, to reach equilibrium, and then filtered to collect the bound [^{3}H]STX for scintillation counting. Non-specific binding was determined in the presence of 10 M TTX, and was generally about 10% of the total binding. The K_d for [^{3}H]STX binding to the synaptosome preparation was found to be about 1.5 nM. TTX inhibited the [^{3}H]STX binding with an IC50 of ~32 nM; using the Chang Prussoff analysis, this corresponds to a K_i of about 6 nM. CNS 1145 was found to inhibit the binding of [^{3}H]STX to synaptosomes with an IC50 of ~22 M, and CNS 1237 inhibited with an IC50 of ~6 M. CNS 1145 was used in further experiments to determine whether the inhibition was competitive or non-competitive. CNS 1145 (20 μM) was found to increase the K_d of [^{3}H]STX binding in competitive manner ($p < 0.02$), with no significant change in the [^{3}H]STX binding site density, indicating a purely competitive mode of inhibition.

Additional [^{3}H]STX binding experiments with several substituted guanidine compounds with either no glutamate release antagonist activity or with glutamate release activity that correlates with inhibition of P-type Ca channels, indicate that they do not affect [^{3}H]STX binding at concentrations of up to 100 μM. This further substantiates the hypothesis that CNS 1145 and its analogues are unusually efficaceous blockers glutamate release, at least in part, due to their ability interact with the SS1-SS2 ion-selectivity filter region of Na channels and/or Ca channels with homologous structures in the SS1-SS2 domain.

Table 1A shows that the listed compounds *competitively* inhibit the binding of [^{3}H]STX, because they decrease the [^{3}H]STX affinity but not the number of [^{3}H]STX binding sites. Graphically this is shown in the accompanying representative Scatchard plot (**Figure 5**), which demonstrates that CNS 1145, 1212, 1237 and 5044 decrease the slope of the binding curve, but not its X-axis intercept.

TABLE IA. Summary of Binding Saturation Experiments Showing Competitive Inhibition of [^{3}H]STX binding by Representative Compounds.

Compound	Concentration of Compound Used#	[^{3}H]STX Affinity (nM) mean sem (n)	No. of [^{3}H]STX Binding Sites (pmol/mg protein) mean sem (n)
Control	≅	1.52 0.10 (6)	3.79 0.29 (6)
CNS 1145	20 M	3.12 0.06 (3)*	3.70 0.37 (2)
CNS 1212	1 M	3.58 0.09 (3)*	3.51 0.26 (2)
CNS 1237	4 M	4.18 0.09 (3)*	3.78 0.49 (2)
CNS 5044	4 M	5.50 0.26 (3)*	3.86 0.24 (2)
CNS 5088	8 M	3.97 0.44 (5)*	3.16 0.34 (4)
CNS 5149	30 M	5.02 0.14 (3)*	3.59 0.37 (3)

This concentration of the indicated compound was present in the binding assay with varying concentrations of [^{3}H]STX.

* Significantly different from Control Kd value; ANOVA $p<0.0001$, followed by the Dunnett multiple comparisons test, $p<0.01$ for each individual compound versus Control.

Table 1B Displays the IC50 values and chemical names for the above compounds and several additional compounds of the invention:

TABLE IB

Inhibition of [^{3}H]Saxitoxin Binding to Na Channels of Rat Brain Synaptosomes.

Example No.	Name	IC50, block of [^{3}H]Saxitoxin Binding, μM	Salt
CNS 1145	N,N'-Bis(5-acenaphthyl)-guanidine	22.0	mesylate
CNS 1209	N,N'-Bis(3-acenaphthyl)-guanidine	3.1	HBr
CNS 1212	N-(5-(3)-acenaphthyl)-N'-(1-anthracenyl)-guanidine	1.4	HCl
CNS 1217	N,N'-Bis(4-tert-butyl-phenyl)-guanidine	18.6	free base
CNS 1222	N-(5-(3)-acenaphthyl)-N'-(4-isopropylphenyl)-guanidine	29.0	HCl
CNS 1237	N-(5-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	5.9	mesylate
CNS 5044	N-(3-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	3.3	HCl
CNS 5088	N-(5-acenaphthyl)-N'-(3-biphenyl)-guanidine	7.2	HCl
CNS 5114	N-(5-Acenaphthyl)-N-(4-ter.butylphenyl) guanidine	7.4	free base
CNS 5118	N-(5-acenaphthyl)-N'-(3-acenaphthyl)-guanidine	8.4	mesylate
CNS 5127	N-(5-acenaphthyl)-N'-(4-chloronaphthyl)-guanidine	14.0	HCl
CNS 5149	N,N'-Bis(4-sec-butylphenyl)-N,N'-bismethyl guanidine	26.0	HCl

Example 6

Inhibition of ^{45}Ca uptake through presynaptic Ca channels:

Compounds of the invention representative of each of the major classes of agents claimed herein were tested to determine their ability to inhibit voltage-activated calcium channels in nerve terminals of mammalian brain. Said voltage-activated calcium channels directly control neurotransmitter release [see Nachsen, D.A. and Blaustein, M.P. (1982) J. Gen Physiol. 79, 1065-1087]. The uptake of ^{45}Ca into brain synaptosomes was performed by an adaptation of the method of Nachsen and Blaustein [*J. Physiol.*, 361:251-258 (1985)], as previously described [Goldin et al., PCT/US92/01050]. The principle of the method involves opening ion permeation through synaptosomal calcium channels by high K^+- induced depolarization of the synaptosomal preparation. The rapid component of ^{45}Ca uptake measured by this procedure is mediated by presynaptic calcium channels.

Briefly, synaptosomes are prepared by the method of Hajos [*Brain Res.*, 93:485-489 (1975)]. Freshly prepared synaptosomes (8 µl) were suspended in low potassium "LK" buffer (containing 3 mM KCl). Test compounds in 8 µl LK buffer were added to synaptosomes to final concentrations ranging from 0.3 µM to 100 µM, and the mixture was preincubated for 5 minutes at room temperature. ^{45}Ca uptake was then initiated by adding isotope in either LK or in buffer ("HK") containing high [potassium] (150 mM KCl). After 5 seconds, the ^{45}Ca uptake was stopped by adding 0.9 ml quench buffer (LK + 10 mM EGTA). This solution was then filtered under vacuum and the filters washed with 15 ml of quench buffer.

Washed filters were subjected to scintillation spectrophotometry to determine the extent of ^{45}Ca uptake. Net depolarization-induced ^{45}Ca uptake was determined for each concentration of each compound tested, as the difference between ^{45}Ca uptake in HK and LK buffers. Results were plotted as % inhibition of depolarization-induced

^{45}Ca uptake vs. [compound] for each compound tested. Representative IC_{50} for inhibition of depolarization-induced ^{45}Ca uptake are presented below in the accompanying tables.

TABLE 2

Inhibiti n of ^{45}Ca uptake through presynaptic Ca channels

Example No.	Name	IC50, block of ^{45}Ca uptake, μM	Salt
CNS 1145	N,N'-Bis(5-acenaphthyl)-guanidine	12	HBr
CNS 1209	N,N'-Bis(3-acenaphthyl)-guanidine	11	HBr
CNS 1217	N,N'-Bis(4-tert-butyl-phenyl)-guanidine	8.2	free base
CNS 1222	N-(5-(3)-acenaphthyl)-N'-(4-isopropylphenyl)-guanidine	6.3	HCl
CNS 1237	N-(5-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	8.9	mesylate
CNS 5044	N-(3-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	9.5	HCl
CNS 5088	N-(5-acenaphthyl)-N'-(3-biphenyl)-guanidine	8.4	HCl
CNS 5114	N-(5-Acenaphthyl)-N-(4-ter.butylphenyl) guanidine	8.1	free base
CNS 5118	N-(5-acenaphthyl)-N'-(3-acenaphthyl)-guanidine	8.6	mesylate
CNS 5127	N-(5-acenaphthyl)-N'-(4-chloronaphthyl)-guanidine	12.3	HCl
CNS 5149	N,N'-Bis(4-sec-butylphenyl)-N,N'-bismethyl guanidine	5.9	HCl

Inhibitory activity of the P/Q-Type Ca channel specific spider toxin, ω-Aga IVa:

Compound	IC_{50}, block of ^{45}Ca uptake, μM
ω-Aga IVa	152 nM

Example 7

Inhibition of ^{45}Ca uptake through L-type (dihydropyridine-sensitive) Calcium Channels:

Compounds of the invention representative of each of the major classes of agents claimed herein were tested to determine their ability to inhibit voltage-activated, dihydropyridine-sensitive L-type calcium channels in clonal GH4C1 pituitary cells. Said voltage-activated L-type calcium channels are found in cardiac muscle, vascular smooth muscle, and the cardiac Purkije cell conduction system (see references in Background and Description of Invention). They are the sites of action of the major classes of Ca antagonists employed to treat hypertension, angina, cardiac arrhythmias, and related disorders. L-type Ca channels are also the sites of action of certain neuroprotective dyhydropyridine Ca antagonists such as nimodipine.

The uptake of ^{45}Ca into GH4C1 cells was performed by an adaptation of the method of Tan, K., and Tashjian, A.H. [*J. Biol. Chem.*, **259**: 418-426 (1984)]. The principle of the method involves activating ion permeation through synaptosomal calcium channels by high K^+- induced depolarization of the synaptosomal preparation. The uptake of ^{45}Ca measured by this procedure is mediated by presynaptic L-type calcium channels, and is sensitive to dihydropyridine, phenylalkylamine, and benzothiazipine Ca antagonists at therapeutically relevant concentrations [Tashjian and Tan, *ibid*, and unpublished data, Cambridge NeuroScience]. The adaptation of the aforementioned method involves growing GH4C1 cells in 96-well culture plates, and is designed to provide a rapid and quantitative determination of the potency of various compounds in inhibiting ^{45}Ca uptake through L-type Ca channels.

Details of method:

GH4 cells, stored in liquid nitrogen, are suspended in 15 ml growth medium (Ham's F-10 medium plus 15% heat-inactivated horse serum and 2.5% heat-inactivated

fetal bovine serum). The cells are centrifuged, resuspended, and then added to T-75 flasks containing 12-15 mls Growth Medium, and incubated at 37°C for approximately 1 week. The cells are them removed from the T75 flask after dissociation from the walls of the flask by treatment for 5 minutes at 37°C with 1 mg/ml Viocase. The Viocase is decanted, and the cells are resuspended in ~ 200 ml of Growth Medium. The cells are then aliquoted (200 µl/well) into each well of several 96 well plates. The cells are then grown under the aforementioned conditions for 3-4 weeks, with replacement of Growth Medium occurring twice per week. Cells are fed growth medium 24 hours before they are employed for ^{45}Ca uptake determinations.

At the time of the assay, media is aspirated from each 96-well plate using a manifold designed to allow 50 µL of liquid to remain in each well. Each plate is washed and aspirated twice with a low K^+ buffer solution "LKHBBS" (in mM 5 KCl, 145 NaCl, 10 Hepes, 1 $MgCl_2$, 0.5 $MgCl_2$, 10 glucose, pH 7.4), 200 µl/well. Each plate is incubated for 10 minutes at 37°C, and aspirated as above. To each well of each plate, 50 µl of HBBS containing the drug to be tested in twice the final concentration is added. The plates are incubated for 10 minutes at room temperature. To each well of each plate, 50 µl of either of two solutions are added:

(a) LKHBBS containing 1 µCi of carrier-free ^{45}Ca, or

(b) HKHBBS (a high K^+ buffer containing 150 mM KCl and no NaCl, but otherwise identical to LKHBBS).

Each plate is then incubated for 5 minutes at room temperature, aspirated as above, and quenched with 200 µl/well of Quench Buffer (Ca-free LKHBBS containing 10 mM Tris-EGTA). Each plate is aspirated and rinsed with Quench Buffer a second time, then carefully aspirated to dryness. To each well of each plate 100 µl of High Safe II scintillation fluid is added. The plates are sealed, shaken, and subjected to scintillation spectrophotometry on a Microbeta 96-well Scintillation Counter (Wallac, Gaithersburg, MD, USA).

Net depolarization-induced ^{45}Ca uptake was determined for each concentration of each compound tested, as the difference between ^{45}Ca uptake in HKBBS and LK buffers. Results were plotted as % inhibition of depolarization-induced ^{45}Ca uptake vs. [compound] for each compound tested. Representative IC_{50} for inhibition of depolarization-induced ^{45}Ca uptake are presented below in the accompanying tables.

TABLE 3

Inhibition by substituted guanidines of ^{45}Ca uptake through L-type Ca channels

Formula I: N,N-DISUBSTITUTED GUANIDINES

Example No.	Name	IC_{50}, block of ^{45}Ca uptake, μM	Salt
CNS 1145	N,N'-Bis(5-acenaphthyl)-guanidine	6.1	HBr
CNS 1209	N,N'-Bis(3-acenaphthyl)-guanidine	9.6	HBr
CNS 1212	N-(5-(3)-acenaphthyl)-N'-(1-anthracenyl)-guanidine	2.0	HCl
CNS 1217	N,N'-Bis(4-tert-butyl-phenyl)-guanidine	4.1	free base
CNS 1222	N-(5-(3)-acenaphthyl)-N'-(4-isopropylphenyl)-guanidine	2.9	HCl
CNS 1237	N-(5-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	2.2	mesylate
CNS 5044	N-(3-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	2.2	HCl
CNS 5088	N-(5-acenaphthyl)-N'-(3-biphenyl)-guanidine	2.2	HCl
CNS 5114	N-(5-Acenaphthyl)-N-(4-ter.butylphenyl) guanidine	2.1	free base
CNS 5118	N-(5-acenaphthyl)-N'-(3-acenaphthyl)-guanidine	5.3	mesylate
CNS 5127	N-(5-acenaphthyl)-N'-(4-chloronaphthyl)-guanidine	3.9	HCl
CNS 5149	N,N'-Bis(4-sec-butylphenyl)-N,N'-bismethyl guanidine	4.3	HCl

Activity of therapeutically effective reference compounds against L-type Ca channels:

Compound	IC_{50}, block of ^{45}Ca uptake, μM (N = # of experiments)
verapamil	8.0 +/- 4, (N = 3)
diltiazem	19.7 +/- 6, (N = 3)

Example 8

Inhibition of ^{14}C-guanidine uptake through Type II Neuronal voltage-activated sodium channels

Antagonists of the neuron-specific type II subclass of voltage-activated Na channels are neuroprotective [Stys, P. K., S. G. Waxman, and B. R. Ransom (1992) J. Neurosci. **12**, 430-439]. The ability of compounds of the invention to block voltage-activated Type II Na channels was determined in a functional assay employing a Chinese Hamster Ovary ("CHO") cell line expressing cloned Type II Na channels derived from rat brain [West, J. W., T. Scheuer, L. Maechler and W. A. Catterall (1992) Neuron **8**, 59-70]. The assay is based on the observation that veratridine, an alkaloid neurotoxin, causes persistent activation of sodium channels, and tetrodotoxin, a heterocyclic agent derived from puffer fish, is a potent and highly specific blocker of several major subclasses of voltage-activated sodium channels, including the said Type II subclass. It further takes advantage of the finding that guanidinium cation will permeate through tetrodotoxin-sensitive Na channels when said channels are opened, either by membrane depolarization [Hille, B., *Ionic Channels of Excitable Membranes 2nd Edition*, Sinauer Associates, Sunderland, MA (1992) pp. 349-353] or by exposure to veratridine [Reith, M.E.A., *Eur. J. Pharmacol.* **188** (1990), 33-41]

Accordingly, the assay entails measuring veratridine-stimulated, tetrodotoxin-sensitive influx of [^{14}C]-guanidinium ion through cloned Type II Na channels expressed in CHO cells. The protocol of the assay is described below.

Assay protocol:

The aforementioned CHO cell line is grown by standard cell culture techniques in RPMI 1640 medium (Media Tech), supplemented with 5% fetal calf serum (Hyclone), 200 µg/ml G418 (Sigma) and 5.75 mg/ml proline (Sigma). Cells are routinely allowed to grow for 3-4 days *in vitro*.

Cultures are rinsed 3 times with 200 l of "preincubation buffer" (5.4 mM KCl, 0.8mM MgSO4, 50 mM Hepes, 130 mM choline chloride, 0.1 mg/ml BSA, 1 mM guanidine HCl, 5.5 mM D-glucose, pH7.4) and incubated with 200 l preincubation buffer at 37°C for 10 minutes. A 96-channel manifold connected is used to vacuum-aspirate the buffer from the wells between rinses.

Different concentrations of CNS test compounds are prepared by dilution into "uptake buffer" (preincubation buffer plus ~2.5 mCi/ml [^{14}C]-guanidinium HCl, ~40 mCi/mmol) containing veratridine (100 M). Aliquots (50 l) of these working stocks is added to the 96-well plates and incubated at room temperature for 1 hour. The veratridine-induced [^{14}C]-guanidinium uptake was linear with time and a good signal (4-8 fold basal uptake) was obtained following a 1 hour incubation. The following controls are also conducted in each 96-well plate: basal uptake (obtained in the absence of CNS compound and veratridine), uptake evoked by veratridine alone, and veratridine evoked uptake in the presence of 10 M tetrodotoxin (the latter is a measure of non-specific uptake independent of Na channel activation).

The flux assay is terminated at the end of the incubation period by rinsing the 96-well plates 3 times with 200 l/well of ice cold "wash buffer" (163 mM choline chloride, 0.8 mM Mg SO_4, 1.8 mM Ca Cl_2, 5 mM Hepes, 1 mg/ml BSA). The remaining 50 l of wash buffer in the wells (following rinsing) is removed by vacuum aspiration with an 8-channel Drummond aspirator. 100 l of High Safe II scintillation

fluid is added to each well. The plates are sealed before shaking for 15 min. The plates are then allowed to sit for 45 min. before counting in a 96-well scintillation counter.

Net veratridine-induced [^{14}C]-guanidinium uptake was determined for each concentration of each compound tested, as the difference between [^{14}C]-guanidinium uptake in the presence and absence of tetrodotoxin. Results were plotted as % inhibition of veratridine-induced [^{14}C]-guanidinium uptake vs. [compound] for each compound tested. Representative IC_{50} for inhibition of veratridine-induced [^{14}C]-guanidinium uptake are presented below in the accompanying tables.

TABLE 4

Inhibition by substituted guanidines of [^{14}C]-guanidinium uptake through type II sodium channels

Code No.	Chemical Structure	IC_{50}, block of ^{14}C guan uptake, μM	Salt
CNS 1145	N,N'-Bis(5-acenaphthyl)-guanidine	2.0	HBr
CNS 1209	N,N'-Bis(3-acenaphthyl)-guanidine	1.0	HBr
CNS 1212	N-(5-(3)-acenaphthyl)-N'-(1-anthracenyl)-guanidine	2.4	HCl
CNS 1217	N,N'-Bis(4-tert-butyl-phenyl)-guanidine	1.5	free base
CNS 1222	N-(5-(3)-acenaphthyl)-N'-(4-isopropylphenyl)-guanidine	0.55	HCl
CNS 1237	N-(5-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	1.3	mesylate
CNS 5044	N-(3-acenaphthyl)-N'-(4-methoxynaphthyl)-guanidine	1.3	HCl
CNS 5088	N-(5-acenaphthyl)-N'-(3-biphenyl)-guanidine	0.5	HCl
CNS 5114	N-(5-Acenaphthyl)-N-(4-ter.butylphenyl) guanidine	1	free base
CNS 5118	N-(5-acenaphthyl)-N'-(3-acenaphthyl)-guanidine	1.3	mesylate
CNS 5127	N-(5-acenaphthyl)-N'-(4-chloronaphthyl)-guanidine	1.1	HCl
CNS 5149	N,N'-Bis(4-sec-butylphenyl)-N,N'-bismethyl guanidine	1.0	HCl

Example 9

Neuroprotective efficacy of compounds of the invention in a rat MCAO model of stroke-induced focal ischemia

The results herein demonstrate the neuroprotective efficacy of a compound of the invention, CNS 1237, in the rat permanent MCA occlusion model of focal ischemia. The results indicate that the compound, dosed i.p. as a single bolus prior to MCA occlusion affords significant neuroprotection with relatively modest effects on blood pressure. The data offer encouragement that we are solidly on track towards development and refinement of a compound series that should give rise to a safe and effective development candidate for limitation of ischemic brain damage.

METHODS:

MCAO model

Focal ischemia was induced in isoflurane-anaesthetized rats by occlusion of the MCA, using the technique of Shiraishi et al. (1989)[1]. Male Sprague-Dawley rats (275-300 g) were induced with 5% isoflurane, intubated, and ventilated with 1.5% isoflurane. The femoral artery and vein were cannulated. Animals were maintained on a heating pad and under a heating lamp. Blood pressure was monitored continuously during and for up to 30 minutes following the surgery for selected animals. An incision was made over the temporal scalp, the temporalis muscle was retracted, and a portion of the maxilla was removed to expose the foramen ovale. The foramen ovale was enlarged to expose the origin of the MCA. The MCA was then coagulated under direct vision from its origin to the olfactory tract. Care was taken to coagulate the penetrating arteries that arise from the MCA and supply the lateral caudate at this level. The

operator was blinded as to which animal received drug. Compound was administered in 0.3 M mannitol as an i.p. bolus 5 minutes before MCA occlusion. An equal volume of vehicle was infused i.v. to control animals at corresponding times.

Infarct size:

Animals were euthanized with 300 mg/kg of pentobarbital at 24 hr after MCA occlusion. Brains were coronally sectioned every at 2 mm intervals from the anterior limit of the caudate to the posterior hippocampus. Sections were rapidly immersed in 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) in buffered Ringer's solution at pH 7.4 for 20 minutes[3] , and sections were then transferred to 4% formaldehyde buffer for 15 minutes prior to photography. Photographs of the 8 sections were then analyzed by a blinded observer using a computerized image analysis system (MCID, St. Catherine, Ontario, Canada). Unstained tissue was classified as infarcted. Hemispheric infarct area in each section was calculated by subtracting the area of normally TTC staining brain in the ipsilateral ischemic hemisphere from the contralateral non-ischemic area. The volume of infarction for the total brain hemisphere was calculated by summing the infarct area in each section measured and multiplying by the distance between sections. This technique minimizes the effect of edema upon measurement of infarct size[11].

Statistical Analysis:

Infarct volumes and physiological variables are expressed as mean +/- S.E.M. for each of the four animal groups: control (N = 6); 6 mg/kg (N = 6); 12 mg/kg (N = 4); and 24 mg/kg (N = 4). The infarct size results were also subjected to One-way Analysis of Variance (AVOVA) and the Bonferroni Multiple comparisons Test to determine P value. Differences between control groups and each dose were regarded as statistically significant (*) if the P value in the latter analysis was < 0.05.

Blood pressure data were of a preliminary nature, and it is premature to subject that data to statistical analysis.

RESULTS:

The infarct size of the control groups in the current study correlates well with those of historical controls done in the same laboratory, exemplified by a recent study of neuroprotection by the Burroughs Welcome glutamate release blocker BW 1003C87.[2]

The neuroprotection data are summarized in **Figure 12**. CNS 1237 delivered as an i.v. bolus, 12 mg/kg right before the MCAO, produced a 32% reduction in infarct volume, as measured by TTC staining. Although there were only 4 animals in that group, the S.E. M. was 7.7% and the data were statistically significant with respect to the control group ($P < 0.05$). The lower dose (6 mg/kg) displayed a comparable degree of neuroprotection: 29% (S.E.M. = 6.3%, $P<0.05$). In contrast, the highest dose, 24 mg/kg, displayed a lower level of neuroprotection that was below the level of statistical significance (16%, $P > 0.05$).

There were no animal deaths at any dose level tested. The operator observed that a subgroup of animals were slower to awaken from anesthesia, and when the code was broken this turned out to correlate in qualitative terms with animals treated with CNS 1237.

Example 10

Neuroprotective efficacy of compounds of the invention: reduction of the volume of lesions induced by chemically-induced depolarization of brain tissue

Glutamate is believed to be the most abundant excitatory neurotransmitter in the brain, and at the elevated levels known to be present in cerebral ischemia, trauma and other pathologic states, is highly neurotoxic. Several major classes of glutamate receptors have been identified including NMDA, AMPA and kainate receptors. Kainate receptors are present in a number of brain regions (including the hippocampus) that are critical for memory function. Numerous investigators have examined the lesions that occur following kainate administration and have determined that kainate's excitotoxic effects are not only due to direct neuroexcitation, but also to enhanced release of glutamate which then generates a neurotoxic cascade. Thus the destructive effects of kainate are reduced by agents (such as lamotrigine) that inhibit the synaptic release of glutamate, and intracranial kainate injection can serve as an assay for identifying more effective glutamate-release blockers.

This example demonstrates the use of a kainate neurotoxicity model to identify compounds with *in vitro* glutamate-release inhibiting properties to determine whether they are neuroprotective *in vivo* and therefore of potential value in the treatment of disorders associated with excessive glutamate release. In brief, young adult rats (150g) are stereotactically injected with 1 g of kainate (in 0.3M mannitol) in the striatum. They are treated with potentially neuroprotective glutamate-release inhibiting compounds that are either co-injected with the kainate (intracranial administration) or injected intraperitoneally. After a seven day survival period, the animals are sacrificed and the brains are removed and stained with triphenyltetrazolium chloride which by

brilliantly staining vital tissue, outlines the lesion site. Computerized image analysis is then used to determine lesion size and the significance of the neuroprotection is determined.

The results demonstrate the ability of three compounds of the invention to significantly reduce lesion size (see **Figure 13**). These are CNS 1237 (16 mg/kg, i.p.) and CNS 5149 and 1217 (i.c. at 4 g/ rat). The results are summarized in the table below.

KAINATE NEUROPROTECTION MODEL

COMPOUND	DOSE	RTE.	LESION VOLUME (mm3) Mean sem (n)	% REDUCTION
Control	≡	i.p.	90.2 7.3 (22)	0
CNS 1237	16 mg/kg	i.p.	49.5 7.8 (8)**	45%
CNS 5149	4 g	i.c.	53.3 3.7 (9)**	41%
CNS 1217	4 g	i.c.	57.2 11.9 (6)*	37%

While not wishing to be bound by theory, the compounds identified with this assay are believed to be neuroprotective as a result of their ability to block glutamate release. These compounds and others of their class have strong potential as therapeutics for the wide range of neurologic disorders in which excessive glutamate release leads to neuronal death and cerebral dysfunction.

CLAIMS

We claim:

1. A compound comprising a substance which is a use dependent blocker of voltage-activated ion channels and interacts with the SS1/SS2 ion selectivity filter site of said ion channels.

2. A compound of Claim 1 wherein said substance is a substituted guanidine.

3. A method of treating a disorder of mammals which is associated with increased frequency, duration or inappropriate timing of depolarization of cells in said mammal comprising the administration to said mammal of a therapeutically effective amount of a compound which is a use dependent blocker of voltage-activated ion channels and interacts with the SS1/SS2 ion-selectivity filter region of said channels.

4. A method of Claim 3 wherein said disorder is caused by decreased oxygen delivery to said cells.

5. A method of Claim 4 wherein said decreased oxygen delivery is caused by a blocked blood vessel or traumatic injury.

6. A method of Claim 3 wherein said cells comprise neuronal, cardiac or endocrine tissue.

7. A method of Claim 3 wherein said mammals are human or equine.

8. A method of Claim 3 wherein said voltage-activated ion channels are calcium or sodium channels.

9. A method of Claim 8 wherein said SS1/SS2 ion-selectivity filter region is comprised of an amino acid sequence including sequences selected from the group of **Figure 14**.

10. A method of treating a neurological disorder of humans which is associated with an increased frequency, duration or inappropriate timing of depolarization of neuronal cells of said human comprising the administration to said human of a therapeutically effective amount of a substituted guanidine which is a use dependent blocker of calcium and sodium ion channels and interacts with the SS1/SS2 ion-selectivity filter of said ion channels.

11. A method of Claim 10 wherein said neurological disorder is stroke, ischemic neuronal damage resulting from cardiovascular surgery, or traumatic brain injury.

12. A method of Claim 11 wherein said guanidine is a di-substituted, tri-substituted or tetra-substituted guanidine.

13. A method for treating a disorder of a mammalian of nervous system in which the pathophysiology of the disorder involves increased frequency, duration or inappropriate timing of depolarization of neuronal cells comprising administering to said mammal exhibiting symptoms of said disorder an effective amount of a compound of Claim 1.

14. A method of treating nerve cell death in a mammal comprising administering to said mammal exhibiting symptoms of nerve cell death or susceptibility to nerve cell death an effective amount of a compound of Claim 1.

15. A method of treating a disease of the cardiovascular system of mammals comprising administering to said mammal suffering from or susceptible to a cardiovascular disease an effective amount of a compound of Claim 1.

16. A method of modulating cellular secretion in a subject comprising administering to said subject an effective amount of Claim 1.

17. A method of treating a disease of mammals in which the pathophysiology of the disease involves inappropriate cellular secretion comprising administering to said mammal suffering from or susceptible to said disease an effective amount of a compound of Claim 1.

18. A method for 1) blocking voltage-activated calcium channels of mammalian neuronal cells; 2) blocking voltage-activated sodium channels of mammalian neuronal cells; 3) blocking voltage-activated calcium channels of mammalian cardiac cells; 4) blocking voltage-activated sodium channels of mammalian cardiac cells; 5) blocking voltage-activated calcium channels of mammalian smooth or skeletal muscle; or 6) blocking voltage-activated sodium channels of mammalian smooth or skeletal muscle cells, comprising administering to said cells an effective amount of a compound of Claim 1.

19. A pharmaceutical composition comprising a therapeutically effective amount of one or more compounds of Claim 1 and a pharmaceutically acceptable carrier.

20. A method of identifying compounds of Claim 1 comprising the *in vitro* testing of compounds for the abilities of said compounds to both: 1) competitively displace suitably labelled ligands which bind to the SS1/SS2 ion-selectivity site of voltage activated ion channels at concentrations which approximate those required to demonstrate biological activity; and 2) effect a use-dependent block of voltage-activated ion channels as demonstrated by electrophysiological methods.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02301

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07C 279/02, 279/04, 279/16, 279/18; A61K 31/155
US CL :514/634; 564/230, 237, 238
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO, A, 92/14697 (GOLDIN ET AL.) 03 SEPTEMBER 1992, see abstract and claims, specifically page 21.	1-2,10-14,18-20 --- 3-9,15-17
X --- Y	WO, A, 91/18868 (KEANA ET AL.) 12 DECEMBER 1991, see abstract and page 28.	1-2,10-15,19 --- 3-9,15-18,20
X --- Y	WO, A, 90/14067 (KEANA ET AL.) 29 NOVEMBER 1990, see abstract, claims and page 11	1-2,10-14,19 --- 3-9,15-18,20
X --- Y	US, A, 5,262,568 (WEBER ET AL.) 16 NOVEMBER 1993, see abstract, claims, column 4, lines 35-52, and column 14, lines 3-20.	1-2,10-14,19 --- 3-9,15-18,20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 JUNE 1995	08 JUN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MARYAC CEBULAK Telephone No. (703) 308-1235

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02301

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 4,906,779 (WEBER ET AL.) 06 MARCH 1990, see abstract, claims, and column 4, lines 35-64.	1-2,10-14,19 ---------- 3-9,15-18,20

INTERNATIONAL SEARCH REPORT

International application No.:
PCT/US95/02301

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

514/634; 564/230, 237, 238
The claims were searched in the above subclasses as much as could be determined from the exemplified compounds in the disclosure.